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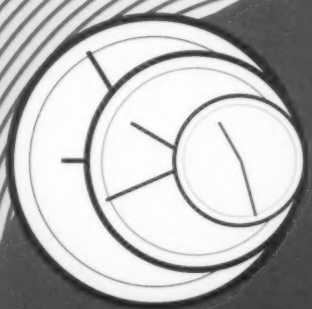
CHRONOBIOLOGY INTERNATIONAL

The Journal of Biological and Medical Rhythm Research



Official Journal of

International Society for Chronobiology

American Association for Medical Chronobiology and Chronotherapeutics

Society for Light Treatment and Biological Rhythms

VOLUME 22/NUMBER 6/2005

Chronobiology International

The Journal of Biological and Medical Rhythm Research

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Chronobiology International (ISSN: 0742-0528) is published six times a year in February, April, June, August, October, and December for a total of 6 times per year by Taylor & Francis, LLC, 325 Chestnut Street, Philadelphia, PA 19106. Periodicals postage paid (Permit no. 016-646) at Philadelphia, PA and additional mailing offices.

US Postmaster: Please send address changes to *Chronobiology International* c/o Taylor & Francis, 325 Chestnut Street, Suite 800, Philadelphia, PA, 19106, USA.

Annual Subscription, Volume 22, 2005

Print ISSN - 0742-0528, Online ISSN - 1525-6073

Institutional subscribers: US $945, UK £573; Personal subscribers: US $238, UK £144

An institutional subscription to the print edition includes free access to the online edition for any number of concurrent users across a local area network.

Production and Advertising Office: 325 Chestnut Street, Philadelphia, PA 19106. Tel - 215-625-8900, Fax - 215-625-8563. Production Editor: Kevin Pickering

Subscription offices

USA/North America: Taylor & Francis LLC, 325 Chestnut Street, Philadelphia, PA 19106. Tel: 215-625-8900, Fax: 215-625-2940.

UK/Europe: Taylor & Francis Customer Services, Sheepen Place, Colchester, Essex CO3 3LP, UK. Tel: +44 (0) 20-7017-5544, Fax: +44 (0) 20-7017-5198.

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Chronobiology International, 22(6): iii–v, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500437136

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FORTHCOMING ISSUE

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7. H. Okamura: Clock genes in mammalian clock cells. (Tentative title)

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Chronobiology International, 22(6): vii–viii, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500498807

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EDITORIAL

The year 2006 will mark the 23rd anniversary of *Chronobiology International*. Since its inception in 1984, the Journal has continually striven to meet the needs of all chronobiologists, regardless of their training and exact interest, from the basic neurobiological, molecular and genetic attributes of central and peripheral clocks to the application of chronobiology to daily life, including occupational (shift work), sport and clinical medicine, and aging, among others. The field of chronobiology has developed rapidly in the past decade, and it is likely to play a central role in future biological discoveries and applications.

During the past two decades, in particular, the amount of research devoted to biological rhythms increased significantly. During this time, *Chronobiology International* also grew in statue and volume. The Journal is currently the official organ of the International Society of Chronobiology, Society for Light Therapy and Biological Rhythm Research, and Association of Medical Chronobiology and Chronotherapeutics. In addition to featuring peer-reviewed original research and in-depth review papers, the Journal publishes abstracts of meetings and congresses organized by these chronobiology groups. The Journal also features special issues organized by renowned guest editors. During the last 5 years, special issues have been devoted to: the functional and adaptive mechanisms of circadian systems, shift work, circadian rhythms and sleep in human aging, and cancer chronotherapeutics. In 2006, the Journal will devote a large special issue to the pineal gland and melatonin.

Over the years the Journal has benefited greatly from the dedication and service of many chronobiologists–as members of the editorial board, as reviewers of submitted manuscripts, and as journal editors. Dr. Reinberg (Paris, France) retired in 1994 from *Chronobiology International* after serving as its co-editor-in-chief for 11 years, and with the completion of the current volume 22 in 2005. Dr. Rensing (Bremen, Germany), who succeeded Dr. Reinberg is now retiring after serving 11 years as co-editor-in-chief of the Journal. I am grateful to Ludger for his strong devotion, outstanding advice and always unselfish contributions to the Journal, field of chronobiology and to me as the senior co-editor-in chief. I will miss his council and collaboration. I am certain he will continue

to be productive in his new endeavors, and I wish him and his family the very best of success and health. Dr. Yvan Touitou (Paris, France) succeeds Dr. Rensing, commencing his term as the new co-editor-in-chief of the Journal in 2006. I eagerly look forward to working closely with Yvan in 2006 and beyond to further build the quality and reputation of the Journal.

Michael H. Smolensky, Ph.D.
Senior Co-editor-in-Chief, *Chronobiology International*
December 2, 2005

Chronobiology International, 22(6): 951–961, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500395011

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HUMAN INTESTINAL CIRCADIAN CLOCK: EXPRESSION OF CLOCK GENES IN COLONOCYTES LINING THE CRYPT

L. Pardini, B. Kaeffer, A. Trubuil, A. Bourreille, and J.-P. Galmiche

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Biological clock components have been detected in many epithelial tissues of the digestive tract of mammals (oral mucosa, pancreas, and liver), suggesting the existence of peripheral circadian clocks that may be entrainable by food. Our aim was to investigate the expression of main peripheral clock genes in colonocytes of healthy humans and in human colon carcinoma cell lines. The presence of clock components was investigated in single intact colonic crypts isolated by chelation from the biopsies of 25 patients (free of any sign of colonic lesions) undergoing routine colonoscopy and in cell lines of human colon carcinoma (Caco2 and HT29 clone 19A). *Per-1*, *per-2*, and *clock* mRNA were detected by real-time RT-PCR. The three-dimensional distributions of PER-1, PER-2, CLOCK, and BMAL1 proteins were recorded along colonic crypts by immunofluorescent confocal imaging. We demonstrate the presence of *per-1*, *per-2*, and *clock* mRNA in samples prepared from colonic crypts of 5 patients and in all cell lines. We also demonstrate the presence of two circadian clock proteins, PER-1 and CLOCK, in human colonocytes on crypts isolated from 20 patients (15 patients for PER-1 and 6 for CLOCK) and in colon carcinoma cells. Establishing the presence of clock proteins in human colonic crypts is the first step toward the study of the regulation of the intestinal circadian clock by nutrients and feeding rhythms.

Keywords Large intestine, colon, per-1, per-2, clock genes, circadian rhythm

INTRODUCTION

The cells of mammals, and most other organisms, reveal circadian rhythms, the molecular mechanism known as the circadian clock. This mechanism regulates many biochemical, physiological, and behavioral processes, including digestion (Harmer et al., 2001), and can be disrupted by social stressors and shifts of the light/dark (LD) cycle like night work and transmeridian flights as well as by serious illnesses such as colon cancer (Mormont et al., 2002; Chevalier et al., 2003). The clock receives an

Submitted June 7, 2004, Returned for revision July 5, 2004, Accepted October 25, 2004
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input signal from environmental cues, and consists of an oscillatory system that generates output signals controlling circadian changes of cellular functions, including the expression of genes (Cermakian and Boivin, 2003).

The mammalian circadian system consists of at least two major oscillatory systems, one that is entrainable by light (central clock located in the SCN) and another (peripheral clock) that is anatomically and functionally distinct but of unknown location (Stephan and Davidson, 1998) and entrainable by food. Stokkan and colleagues (2001) have shown that feeding cycles in transgenic rats could entrain the clock of liver independently of the suprachiasmatic nuclei, and according to Davidson and coworkers (2003) the gastrointestinal system is a possible site for a main peripheral clock. However, the existence of a circadian clock in intestinal epithelial cells is still unexplored, although intestinal circadian rhythms have been described in cell proliferation (Goodlad and Wright, 1984; Buchi et al., 1991; Marra et al., 1994), migration (Qiu et al., 1994), differentiation (Saito et al., 1978), and death (Ijiri & Potten, 1988, 1990). It is important to establish the connections between the intestinal biological clock and the timing of epithelial cell-cycle events (Bjarnason and Jordan, 2002) in order to improve colon cancer chemotherapy (Lévi, 2002) and to study the influence of food intake on gut physiology (Stephan and Davidson, 1998; Davidson et al., 2003).

The intracellular oscillatory system generates rhythms through the expression of several human clock genes, including *per-1*, *2*, and *3*, *clock*, *bmal1*, *cry-1* and 2, and *casein kinase 1-epsilon*, which are interconnected by positive and negative feedback loops (Piggins, 2002). The main activator complex is a heterodimer of two transcription factors (CLOCK and BMAL1) bearing a Helix-Loop-Helix/Per-Arnt-Sim (HLH/PAS) domain. The CLOCK:BMAL1 dimers activate the transcriptions of *per* and *cry* genes by binding to E-boxes (Muñoz et al., 2002). The main regulators of this complex are PER-1 proteins that destabilize CLOCK:BMAL1 dimers in the nucleus and PER-2 proteins that activate *bmal1* gene transcription by an unknown molecular intermediate. Clock proteins show a strong similarity in both rodents and humans according to amino acid sequence analysis (Tei et al., 1997; Steeves et al., 1999; Hida et al., 2000). We focused on the best described clock genes (*per-1*, *per-2*, *clock*, and *bmal1*) in crypt colonocytes isolated from biopsies of patients free of any sign of colonic lesions and in two well known colon carcinoma cell lines: Caco2 (Pinto et al., 1983) and HT-29 clone 19A (Augeron and Laboisse, 1984).

MATERIALS AND METHODS

The research followed the ethical guidelines established by the Journal for the study of human biological rhythms (Touitou et al., 2004).

Patients

Two mucosal biopsies (4 to 6 mg wet weight) were taken from the left colon of 25 diurnally active patients (males and females, 25 to 83 yrs of age) who underwent a colonoscopy as part of a gastroenterological work-up. All patients received an oral electrolyte bowel preparation the evening before the colonoscopy. Clinical examination did not reveal any colonic lesions (American Society for Gastrointestinal Endoscopy, 2001). All sampling times were recorded and expressed as circadian time.

Colonic Crypt Preparation

Colonic crypts were extracted from biopsies by chelation (method adapted from Whitehead et al., 1987). Biopsies were collected in 1 mL phosphate-buffered saline solution without Ca^{++} and Mg^{++} (PBS0). The supernatant was removed and replaced by 1.5 mL PBS0 containing 3 mM ethylenediaminetetraacetic acid (Sigma, Saint Quentin Fallavier, France) and 5 mM dithiothreitol (Sigma) for 60 min. Biopsies were rinsed and then exposed to 1 mL PBS0. Crypts were extracted by vigorous shaking. Stromal tissue was removed. Crypts were pelleted by centrifugation. We obtained between 400 and 1000 isolated crypts free of stromal tissues per biopsy in order to perform either real-time reverse transcription and polymerase chain reaction (RT-PCR) or immunostaining. One sample was collected for microdissection with a direct fixation step in PBS0 with 10% formaldehyde followed by ethanol permeabilization (Konishi et al., 1996).

Cell Lines

Colon carcinoma cells (Caco2, ECACC n°8601202), HT-29 clone 19A were kindly donated by Dr. J-P Segain, INRA-Nantes, and were cultured in DMEM medium; whereas, neural tumor cells (Kelly, ECACC n°92110411) were cultured in RPMI-1640 medium, both supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM) and penicillin-streptomycin (Invitrogen). Confluent cell monolayers were used to assay RT-PCR reagents and to standardize immunoreagents.

Real-Time Quantitative RT-PCR

Total RNA of crypts and cell monolayers were extracted in 1 mM of Trizol (Invitrogen, Cergy Pontoise, France). Two μg of each sample were denatured at 70°C (5 min) together with 1 μg of random primers (Promega, Charbonnières, France). Reverse transcription then occurred for 60 min at 37°C and 5 min at 95°C with 10 mM deoxynucleotides and 2.5 μL AMV (Promega). An aliquot of 4.5 μL of the RT reaction was used for quantitative PCR in the presence of 250 nM specific forward

and reverse primers designed on the basis of published data on human homologues of the clock genes in GenBank using Beacon gene designer software (Table 1) and 7.5 μL Sybrgreen Qiagen buffer (Qiagen).

Immunocytochemistry and Confocal Imaging

Crypts and cell monolayers were fixed in PBS0 with 4% formaldehyde overnight at 4°C to preserve their morphology. The next day, the fixative was replaced by ethanol (70%) for permeabilization. An alternative fixation procedure that exposed cell monolayers to cold methanol (−20°C) was tested (Zanello et al., 2000). The samples were rehydrated by progressive exposure to PBS0 with 0.2% bovine serum albumin (Fraction V, Sigma). Anti-PER-1, anti-PER-2, anti-CLOCK, and anti-BMAL1 antibodies (Table 2) were used (1/10 dilution). Specificities of polyclonal antibodies were checked by exposure to peptide inhibitors and by ELISA performed with fixed cell monolayers. Tetramethylrhodamine isothiocyanate (TRITC) conjugated rabbit anti-goat secondary antibodies (Sigma) and epidermal growth factor receptor (EGF-R) monoclonal antibodies were used in 1/50 dilution (Table 2). Nuclear DNA was stained by Hoechst 33258 (50 μg/mL). Crypt samples were mounted in glycerol and observed with a Zeiss LSM 410 confocal microscope.

Data Analysis

RT-PCR data are reported as C_T (number of PCR cycles required for the fluorescence signal to reach a detection threshold) related to the C_T of beta-actin transcripts. Series of confocal images were stored in a WEB-based image collection using the Scientific Image Database

TABLE 1 Human Primer Sequences Used in Real-Time Quantitative RT-PCR (R reverse, F forward) and Determined Using GenBank Database and Beacon Designer Software

Gene	Accession number	Nucleotides	Sequence
per-1	AF022991	R 412-431	5′-AGGCAACGGCAAGGACTCAG-3′
		F 516-537	5′-AGGAGGCTGTAGGCAATGGAAC-3′
per-2	NM_003894	R 646-667	5′-TGGAGGAGATGGAGAGCGTTAC-3′
		F 761-782	5′-GGATGCAACCTGGTCAGAGATG-3′
Clock	AF011568	R 2823-2844	5′-GTCTCAGCAACAGCAGCAACTC-3′
		F 2942-2961	5′-GCCATCCCCTTCCTCCCTTG-3′
egf-r	NM_005228	R 2013-2031	5′-TGTGCCACCTGTGCCATCC-3′
		F 2145-2164	5′-ACCACCAGCAGCAAGAGGAG-3′
beta-actin	NM_001101	R 37-58	5′-TGCTATCCAGGCTGTGCTATCC-3′
		F 177-194	5′-GCCAGGTCCAGACGCAGG-3′

TABLE 2 Antibody Specificities

Antibody	Source	Type	Specificity
PER-1	Tebu, Santa Cruz Biotechnology Le Perray en Yvelines, France	C-20 sc-7723	Affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of human PER-1
	Tebu, Santa Cruz Biotechnology Le Perray en Yvelines, France	N-20 sc-7724	Affinity purified goat polyclonal antibody raised against a peptide mapping at the amino terminus of human PER-1
	Euromedex Souffelweyersheim, France	P-3342-10	Affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of human PER-1.
PER-2	Tebu, Santa Cruz Biotechnology Le Perray en Yvelines, France	N-19 sc-7728	Affinity purified goat polyclonal antibody raised against a peptide mapping at the N-terminus of human PER-2
CLOCK	Tebu, Santa Cruz Biotechnology Le Perray en Yvelines, France	M-20 sc-6928	Affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of mouse CLOCK
BMAL1	Tebu, Santa Cruz Biotechnology Le Perray en Yvelines, France	N-20 sc-8550	Affinity purified goat polyclonal antibody raised against a peptide mapping at the N-terminus of human BMAL1
EGF-R	Sigma Saint Louis, Missouri, USA	E-2760	Monoclonal antibody anti-EGFR prepared from paraformaldehyde-fixed carcinoma cell line A431

system (http://wwwsidb.sourceforge.net). Biomarker fluorescence intensities were qualitatively and quantitatively analyzed by our image analysis software Quant3D (Kaeffer et al., 2002). The variation of fluorescence intensities depending on colonic crypt axis and patient was analyzed using one-way and two-way variance analysis with a 0.95 confidence interval.

RESULTS

Clock Genes are Expressed by Colonocytes

All biopsies were sampled between 10:00 and 13:30 h, and the isolated crypts fixed between 11:00 and 14:30 h in patients fasting for 16 to 19 h. Crypt labeling with different anti PER-1 antibodies resulted in similar distributions, and competitive inhibition assays indicated that both were directed to similar antigen sites on Caco2 cell monolayers (results not shown). Incubations of anti-PER-1 antibodies with corresponding

peptide inhibitors resulted in 80% inhibition of the signal. We demonstrated the presence of *per-1*, *per-2*, and *clock* transcripts in 5 RNA samples prepared from the colonic crypts (Figure 1). PER-1 and CLOCK proteins were immunodetected (Figure 2) in the crypts of 15 (13 with C-terminal antibodies and 2 with N-terminal antibodies) and 6 patients, respectively. PER-2 and BMAL1 were not detected. *Per-1*, *per-2*, *clock*, and epidermal growth factor-receptor (*egf-r*) mRNA were detected in Caco2, HT-29, and Kelly cell lines (Figure 3) after exposure to 50% FCS for 2 h (serum shock, results not shown) as described by Balsalobre and colleagues (1998) and maintained in media without FCS until RNA extraction 16 h after shocks in order to be as close as possible to the physiological conditions of the biopsies. The C_T ratio of the different transcripts was similar in Caco2, HT29, and patients, but it was different for the Kelly

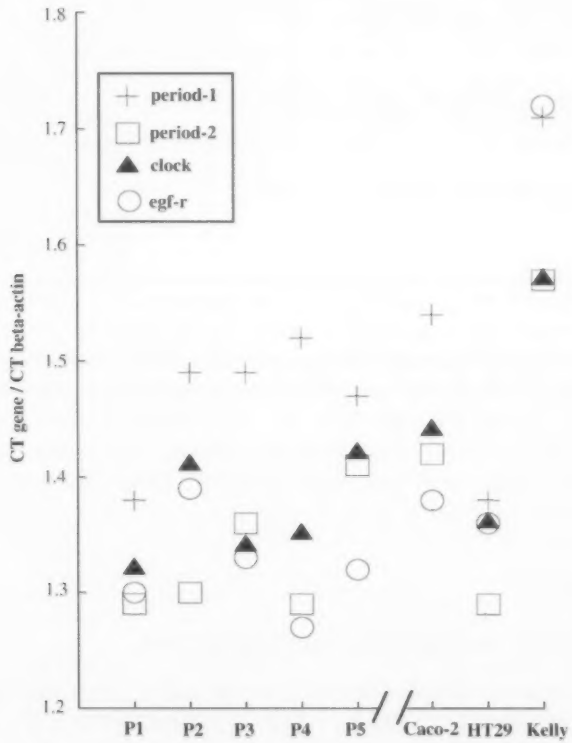

FIGURE 1 Expression of *per-1*, *per-2*, *clock*, and *egf-r* transcripts in RNA samples of intact single colonic crypts (5 healthy patients referred to as P1 to P5 and sampled from 11:05 to 13:25 h) and in Caco-2, HT29 clone 19A, and Kelly cells. RT-PCR data are presented in relation to *beta-actin* gene expression.

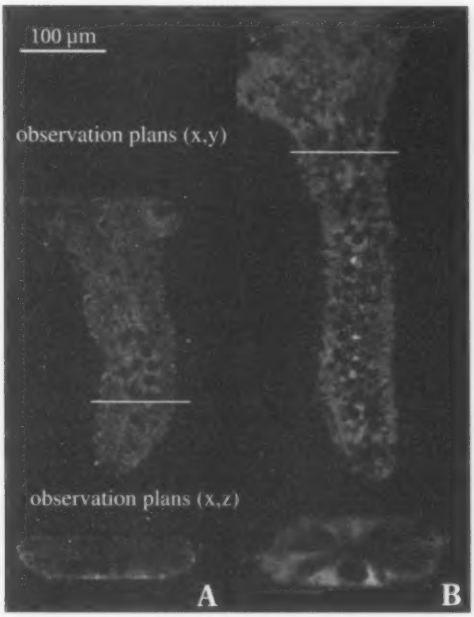

FIGURE 2 Immunostaining of human colonic crypts for PER-1 (A) and CLOCK (B) proteins. Representative crypts shown here were combined reconstructions of 2 confocal micrographs per crypt. They were double labeled for clock proteins by antisera detected by rabbit anti-goat antibodies tagged with TRITC (shown here in gray) and for nuclear DNA by Hoechst 33258 (not shown). Coincident staining indicated intranuclear PER-1 and CLOCK (not shown). Orthogonal electronic sections of crypt axis (optical zoom following white line) are slightly flattened because of the crypt mounting method.

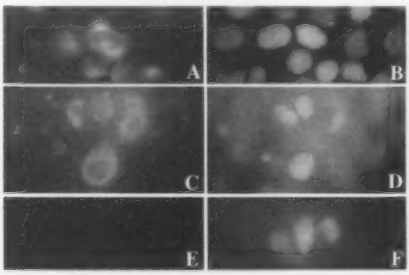

FIGURE 3 Immunostaining of PER-1 (A) and CLOCK (C) proteins in colon carcinoma Caco-2 cells grown on a glass slide, fixed after serum induction, and double labelled for clock proteins (*first column*) and DNA (*second column*). PER-1 (A) immunostaining is perinuclear and not polarized. Some cells are not labelled for PER-1. Negative control shown in E. All observations were performed by epifluorescent microscopy under oil-immersion (×100).

neuroblastoma cells (Figure 1). PER-1 and CLOCK were detected by immunofluorescence in all the cell lines tested (data not shown). PER-2 and BMAL1 were not detected by immunofluorescence in routine culture of Caco2, HT29, and Kelly cells, but they were induced to a detectable level after a serum shock (results not shown).

3D Distributions of PER-1 and CLOCK in Epithelial Cell Lining the Colonic Crypt

PER-1 expression was heterogeneous all along the colonic crypts analyzed (Figure 2A) and especially perinuclear and polarized from stem cells to mature compartments (Figure 4A); whereas, in the senescent compartment, PER-1 was depolarized, but mostly perinuclear (Figure 4C). We did not find any polarization of the PER-1 signal on 7-day-old cell monolayers (Figure 3A). CLOCK distribution was very heterogeneous (nuclear and cytoplasmic) and not polarized (Figure 4E). EGF-R was used as a cell surface marker, and its expression along the colonic crypts of 5 patients was polarized and homogenous (data not shown). Fluorescence intensities were evaluated on 64 crypt profiles for PER-1 and on 18 profiles for CLOCK. The level of expression of PER-1 and CLOCK varied among the patients ($p < 0.001$) and along crypt axis ($p < 0.001$) for all patients.

DISCUSSION

We found *per-1*, *per-2*, and *clock* transcripts in colonocytes isolated from non-cancerous patients fasting for 16 to 19 h. In mammals, the *per-1* level is

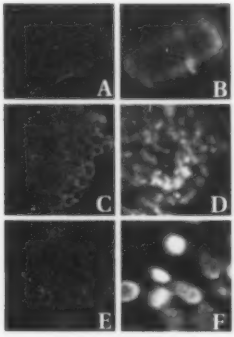

FIGURE 4 Immunostaining of human crypt colonocytes for PER-1 and CLOCK proteins. Crypts were double labelled for clock proteins (first column) and nuclear DNA (second column). Repartitions of PER-1 are displayed at the base (A, B; orthogonal section) and at the apex (C, D) of crypt (zoom of confocal micrograph, ×40). Repartitions of CLOCK are shown in E with the nuclear staining in F (zoom of confocal micrograph, ×100).

shown to be maximal around the middle of the day in the suprachiasmatic nuclei and low during the night (Cermakian and Boivin, 2003). In peripheral tissues of *per-1*-luciferase transgenic rats, the *per-1* level is considered to be delayed by several hours (Oishi et al., 1998; Zylka et al., 1998; Cermakian et al., 2001). The *per-1* expression in human oral mucosa (Bjarnason et al., 2001) shows two maxima: one in the morning (around 08:00 h) and another in the evening (around 20:00 h); whereas, in human colonocytes we observed the lowest C_T for patient P1 sampled at 11:05 h and all other C_T ratios in a close range for patients P2 to P5 sampled from 11:35 to 13:25 h (Figure 1). Further study of the *per-1* variation over the 24 h is needed, but we may infer that the circadian expression of *per-1* may follow a wave along the digestive tract (from oral mucosa to rectum) as described by Scheving (1983) for cell proliferation in the intestinal tract of the mouse. Relative quantification of biomarker fluorescence intensities was applied to detect any differences in the expression of PER-1 and CLOCK proteins along the crypt cellular compartments. We found a constant depolarization of PER-1 in the upper part of the colonic crypts corresponding to the senescent compartment, but we could not demonstrate any link between this loss of polarization and the activation of the senescent program of the colonocytes.

PER-1 and CLOCK proteins were found in two types of colon carcinoma: Caco2 with a fetal enterocyte phenotype and HT29 clone 19A with a phenotype of proliferative colonocytes. There was no striking difference between normal and cancerous cells (Figure 1). Both cell lines expressed these clock transcripts and proteins with a strong inducibility after a serum incubation leading to circadian rhythms (unpublished experiments). The expression pattern of Caco2 and HT29 genes was different from the one observed in the Kelly cell line, suggesting tissue specificity in the circadian expression of these transcripts. In conclusion, this work demonstrates the expression of clock gene transcripts and proteins in healthy and cancerous colonocytes.

ACKNOWLEDGMENTS

Part of this work was presented by Pardini et al. (2003) at the 1st World Congress on Chronobiology, September 9–12, Sapporo (Japan) and funded by a travel grant. We are grateful to Pierre de Coppet for his help and advice in RT-PCR and Jocelyne Coutret for tissue culture assistance (both from INRA-Nantes). Lissia Pardini was a recipient of a Ph.D. grant (Contract B4015 between the region Pays de la Loire, France, the EU and two INRA departments, Biométrie and Nasa).

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Chronobiology International, 22(6): 963–974, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500395086

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MASKING IN *WAVED-2* MICE: EGF RECEPTOR CONTROL OF LOCOMOTION QUESTIONED

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It has been suggested that epidermal growth factors (EGF) are responsible for the inhibition of locomotion by light (*i.e.*, masking) in nocturnal rodents (Kramer et al., 2001). The poor masking response of *waved-2* ($Egfr^{wa2}$) mutant mice, with reduced EGF receptor activity, was adduced in support of this idea. In the present work, we studied the responses to light over a large range in illumination levels, in a variety of tests, with pulses of light and with ultradian light-dark cycles in $Egfr^{wa2}$ mutant mice. No evidence suggested that normal functioning of epidermal growth factor receptors was required, or even involved, in masking.

Keywords Circadian rhythm, Epidermal growth factor, Locomotion, Masking, Subparaventricular zone, *Waved-2*, Mutants

INTRODUCTION

Light exerts a major influence on rhythms of sleep and wakefulness in mammals. Two processes, one direct and one indirect, are involved. The direct route (called masking) mediates acute effects such as the suppression of locomotion in nocturnal species in the presence of light (Aschoff, 1960). The indirect route (called entrainment) is the synchronization by light of an endogenous clock which in turn controls sleep and wakefulness.

Kramer and colleagues (2001) have suggested that both routes depend on epidermal growth factor (EGF) receptor signaling in the subparaventricular zone (SPVZ) of the hypothalamus. Transforming growth factor-alpha (TGFA) from the SCN, by binding to EGF receptors, would mediate clock-controlled inhibition of activity, and either TGFA or EGF,

Submitted August 24, 2005, Returned for revision September 02, 2005, Accepted September 09, 2005

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by a direct projection from the retina, would mediate acute inhibition of activity. The latter point was based on a lack of acute inhibition of locomotion (*i.e.*, a lack of masking) in *waved-2* ($Egfr^{wa2}$) homozygous mice that have dysfunctional ligand-stimulated activity of EGF receptors (EGFR).

This is a potentially elegant and unifying account for the control of locomotion by light. However, examination of the data presented in Kramer and colleagues (2001) reveals a number of potential problems. With respect to the control from the SCN, the lowest TGFA mRNA signal in the SCN of hamsters occurs at circadian time (CT) 18; by then, the activity of hamsters has usually slackened. If TGFA inhibits activity by binding to EGF receptors, one would have expected the lowest signal to be at CT 12 and in the early subjective night, when locomotion is most pronounced (CT 12 by definition is the onset of locomotion). Van der Zee and coworkers (2005) have studied the profiles of TGFA immunoreactivity in the SCN of mice over the 24 h span. High variability and apparent peaks just before dark onset do not support in any obvious way the idea that TGFA inhibits locomotion.

With respect to the direct or masking route, the sharp cessation of activity at "lights on" in Figure 4A of Kramer and associates (2001) hints at the presence of a direct response to light. Moreover, in mice with defects in EGFR function, locomotion during a light pulse was still suppressed somewhat (53% inhibition of its baseline level), even though this was less than in wild-type animals (95% inhibition). This partial response does not justify the strong statement that "EGFR signaling is required for masking." In addition, from Figure 4B of Kramer and colleagues (2001), it looks as if some of the pulses started well before the animal might have become active anyway; to the extent that is so, it would not be optimal for standardized testing of the effect of a variable on suppressing locomotion. Masking of locomotion does vary with the time of day in some circumstances (Redlin and Mrosovsky, 1999). Finally, only a limited range of illumination was studied. This also makes it legitimate to question statements such as "EGFR activity is required for the acute inhibition of activity in response to light."

For these reasons, together with a past interest in masking (Mrosovsky, 1999; Redlin, 2001), we further investigated the role of EGFR. We did not aim to replicate the work of Kramer and coworkers (2001) in every detail, but rather to undertake experiments that we felt would be maximally instructive about the presence or absence of masking in EGFR mutant mice. In the first experiment we asked whether masking could be obtained in $Egfr^{wa2}$ homozygous mutants if sufficient levels of illumination were used. In the second experiment, we looked for evidence of quantitative differences in the amount of masking compared to wild-types and heterozygotes.

METHODS

Animals and Housing

Male *waved-2* (B6EiC#Sn-a/A-*Egfr*^{wa2} *Wnt3a*^{vt}) mice were obtained from the Jackson Laboratory. They were put in L:D 16:8 h with ~750 lux in the L part of the cycle. Initially they were kept in cages without running wheels for 15 (Experiment 1) or 30 (Experiment 2). They were then housed in polypropylene cages 44 × 23 × 20 cm equipped with a running wheel (17.5 cm diameter) for a further 9 (Experiment 1) or 28 (Experiment 2) before tests begun. When the first masking tests were run, the mice in Experiment 1 were 71 ± 16 d old and in Experiment 2, 120 ± 18 d old. At the time of the last test, the animals in Experiment 1 were 137 ± 16 d old and in Experiment 2, 256 ± 18 d old. Wheel revolutions were recorded with Dataquest III hardware and software in 10 min bins. Purina Mills #5001 chow and water were provided *ad libitum*. Room temperature was 20.5 ± 3.5°C. The care and handling of the animals as well as the conduct of experiments adhered to the ethical standards of the Journal (Touitou et al., 2004).

Genotyping

Homozygous *Egfr*^{wa2} mice were immediately distinguishable by their wavy coats and curly whiskers. Nonetheless, all mice were molecularly genotyped as confirmation. Ear or tail snips (<0.5 cm) from each mouse were heated in 0.5 mL of 25 mM NaOH/0.2 mM EDTA for 5 min at 95°C, then neutralized with 5 μL of 1 M HEPES. One microliter of each sample was used in a PCR reaction with *Egfr* specific primers (primers: 5′–TACCCAGAAAGGGATATGCG-3′ and 5′-GGAGC-CAATGTTGTCCTTGT-3′). PCR products were digested with *Fok* I before electrophoresing on a 1% agarose gel. Uncut 230 bp bands were diagnostic for wild-type *Egfr*, while cut 130 bp and 100 bp fragments were specific for the *Egfr*^{wa2} allele of *Egfr*. Heterozygous mice were identified by the presence of all 3 fragments.

Methods of Measuring Masking

Two types of test were used:

1. *Pulses of light in the night*. The apparatus, with separate lights for entraining and for masking pulses, has been described elsewhere (Mrosovsky et al., 1999). As before, illumination was varied in different tests by addition of neutral density filters below three masking lights (Sylvania Octron 4100 K 32 W fluorescent tubes). A Hagner E2X meter was used to provide approximate measures of lux.

Tests were usually separated by 3 d, with one of the intervening days for changing the filters and any other necessary disturbance, and the other intervening day for the baseline; occasionally extra intervening days occurred for logistic reasons. In cases of missing data resulting from technical problems, replacement tests for the relevant illumination levels were run after the main tests ended. Tests were not run in order of increasing or decreasing light levels during the pulses, but in a quasi-random order; however, this precaution was probably unnecessary, as order effects in this type of experiment are absent or minimal (Edelstein and Mrosovsky, 2001). A masking score for each animal in each test was calculated by comparing the amount of wheel running during the pulse with that over the same time on the previous night, and expressing the result as a percent. Thus 100% indicated no masking, and 0% indicated total suppression of activity by the light pulse.

2. *Ultradian LD cycle test.* Mice were kept for at least 1 wk on a L:D 3.5:3.5 h cycle. This test for masking was developed as a way to greatly reduce or eliminate entraining effects of light on the scores for nocturnal activity (Redlin and Mrosovsky, 1999). To appreciate this, one may consider what could be inferred from use of cycles such as L:D 12:12 or 16:8 h. If animals on such schedules ran mostly in the night, this activity might have resulted either from entrainment of an endogenous oscillator, or from a direct effect of light in the L part of the cycle inhibiting activity, or from a combination of both. To study masking one needs procedures that cannot be interpreted as entraining effects of light, just as to study entrainment one needs procedures that eliminate interpretations based on masking. A 3.5:3.5 h L:D cycle makes a 7 h 'day,' $7 \times 3 = 21$ h and $7 \times 4 = 28$ h. Both the 7 h 'day' and simple multiples of 7 h 'day,' are outside the range of entrainment of mammals studied in common laboratory circumstances (see Gorman et al., 2005, for special circumstances). Therefore, the activity distribution in a 3.5:3.5 h cycle reflects masking rather than entraining effects of light. An advantage of this procedure over pulse tests is that the masking is based on behavior throughout a whole week, rather than just on comparison of the activity during a 1 or 3 h pulse period with that on the previous day. The percent of the total activity during the wk that occurred in the dark was used as a measure of masking. Thus, on this test a 50% score means that locomotion was equally likely in the dark and the light 3.5 h periods, and that no masking was present, while a 100% score indicates that no activity occurred in the light, and that activity then was totally masked by light.

Description of Experiments

Experiment 1. The aim of this experiment was to learn whether *Egfr*^{wa2} homozygous mice are capable of strong suppression of locomotion in the

presence of light (*i.e.*, negative masking). Eight $Egfr^{wa2}$ mutant and 7 heterozygous mice were tested; wild-type mice were not available at that time and in any case were not essential for the aim of this experiment. Pulses of light started at zeitgeber time (ZT) 13. By definition ZT = 12 is dark onset. First, 1 h light pulses of different illuminations were tested. Then 3 h pulses, also starting at ZT 13, were given.

Experiment 2. The aim of this experiment was to assess if masking responses to light differed in $Egfr^{wa2}$ mutant mice from those of heterozygous and wild-type mice. In this experiment, the only pulses given were 3 h long. However, masking scores for the first hour out of the 3 h pulse were analyzed separately, as if the pulses had ended after 1 h. Pulses started at ZT 14. All data from 1 homozygous, 1 heterozygous, and 3 wild-type mice were excluded because of low erratic baseline wheel running. This left group sizes of 7, 8, and 4, respectively.

After tests with pulses were finished, the animals were placed on the 3.5:3.5 h L:D schedule. The approximate illumination for the first 14 d was 100 lux in the L portion, for the next 14 d it was 11 lux, and for the final 13 days it was 1.7 lux. Only data from the last wk of each of these 3 periods were analyzed. For this part of the experiment, some of the animals that used the wheel only relatively occasionally could be included, as the scoring method does not depend on comparisons to running in baseline periods. Accordingly, some group sizes (see Table 1) were slightly larger than for the light-pulse experiments; any animals making fewer than 3000 revolutions in the wk remained excluded to minimize potential distortion of day/night distributions by entries into the room for maintenance.

The main differences in our tests compared to those of Kramer and colleagues (2001) were:

1. We studied pulses of light in entrained animals, so that the pulses for all animals would occur at the same circadian time—a time in the night when activity was to be expected.

TABLE 1 Means (±SEMs) of % of Wheel Running Occurring in the Dark Out of the Total Wheel Running During 1 wk Under LD 3.5:3.5 h. Score of 50%, No Evidence of Masking

Lux	*Waved*-2-/− $n = 7$	*Waved*-2-/+ $n = 9$	Wild-type $n = 6$
100	72.9 (±5.5)	81.5 (±4.5)	69.9 (±5.5)
11	64.1 (±4.2)	64.7 (±4.7)	60.9 (±4.4)
1.7	59.5 (±6.2)[a]	53.6 (±4.9)	51.2 (±2.4)

[a] $n = 6$.

2. We employed a range of illuminations, going from levels that gave strong suppression of activity in controls (*i.e.*, negative masking) to dim lights that resulted in increased activity (*i.e.*, positive masking). For terminology, see Mrosovsky (1999).
3. In addition we studied locomotion on a special ultradian light-dark (L:D) cycle designed to measure the masking effect of light with minimal contamination from the entraining effect of light.

RESULTS

Experiment 1

With 1 h pulses of 1700 lux light, locomotion was greatly suppressed (to <20% of baseline, Figure 1). With the 3 h pulses, the effect was almost as large (Figure 2). These results are sufficient to demonstrate that strong masking can be obtained in these mutants. With a repeated measures 2-way analysis of variance (ANOVA) (Statistica software), using genotypes and light level as factors, there was no significant difference between $Egfr^{wa2}$ mutant mice and heterozygotes, either for the 1 h pulses ($p = 0.55$) or for the 3 h pulses ($p = 0.75$).

With the 3 h pulses of light, the activity was not inhibited as strongly toward the end of the pulse as initially when the light came on (Figure 3). To assess this quantitatively, the data for the 3 h pulses were divided into 6 consecutive 30 min time bins. A repeated measures 2-way ANOVA, performed separately for each of the 3 light levels, did not detect any significant differences between the groups (genotype and

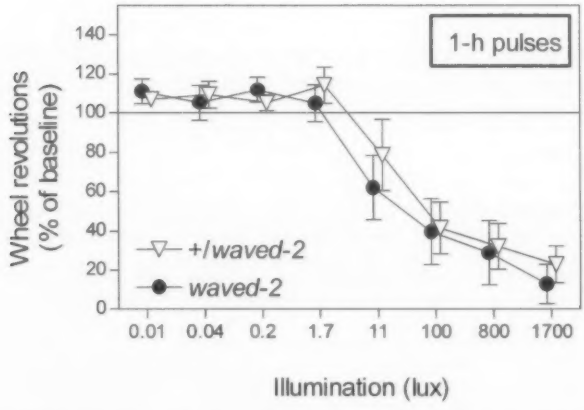

FIGURE 1 1 h pulses in Experiment 1. Means ± SEM for suppression of locomotion during 1 h light pulses of different illumination levels in homozygous and heterozygous $Egfr^{wa2}$ mutants.

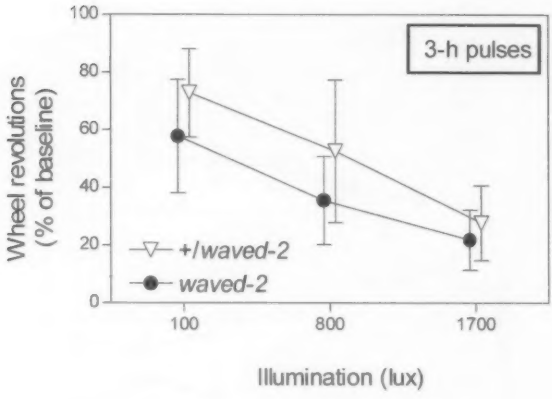

FIGURE 2 3 h pulses in Experiment 1. Conventions same as in Figure 1.

30 min time bin as factors, 1700 lux: $p = 0.48$; 800 lux: $p = 0.73$; 100 lux: $p = 0.44$) and no significant interactions between factors (1700 lux: $p = 0.68$; 800 lux: $p = 0.25$; 100 lux: $p = 0.74$).

Experiment 2

The results of Experiment 2 (Figures 4 & 5) with pulses were similar to those of Experiment 1. With a repeated measures 2-way ANOVA (genotype and light level as factors), there were no significant differences between the genotypes either when the first hour of the pulse was scored ($p = 0.20$), or when the full 3 h was scored ($p = 0.23$).

To learn if there were differences between the genotypes with respect to how long activity remained suppressed by light, separate analyses for each light level from 1700 to 1.7 lux were performed (Figure 6). Repeated measures 2-way ANOVA did not give any significant p values (genotype and 30 min time bin as factors, 1700 lux: $p = 0.41$; 800 lux: $p = 0.34$; 100 lux: $p = 0.08$; 11 lux: $p = 0.33$; 1.7 lux: $p = 0.15$). Interactions between the factors were not significant at the following light levels: 1700 lux ($p = 0.22$), 800 lux ($p = 0.55$), 100 lux ($p = 0.40$), 1.7 lux ($p = 0.61$). However, at 11 lux, a significant interaction was obtained ($p = 0.012$), indicating that in the case of the pulse at this light level the time course of the masking effects was different among the genotypes.

For the tests with the 3.5:3.5 h L:D cycle (Table 1), analysis with a 1-way ANOVA did not reveal any significant differences in the percent running in the dark periods at any of the 3 light levels employed: 100 lux ($p = 0.22$), 11 lux ($p = 0.83$), 1.7 lux ($p = 0.53$).

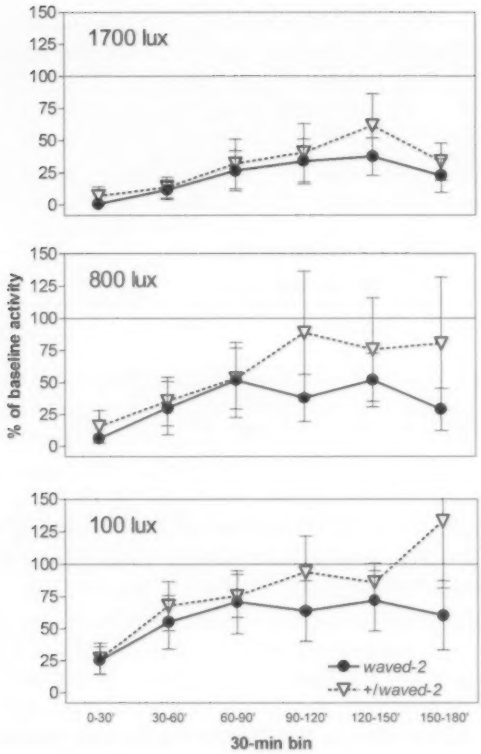

FIGURE 3 Time course of suppression of locomotion in 3 h pulses in Experiment 1. Data were divided in 30 min time bins. Means ± SEM shown.

DISCUSSION

Egfr^{wa2} mutant mice can show strong negative masking if lights are made sufficiently bright, in the order of 1000 to 2000 lux. Even without any comparison groups, it is clear that normal EGFR functioning is not required for masking. We obtained only two possible, but weak, hints of impaired masking.

First, although the overall ANOVA in Experiment 2 of the data for the first hour of the pulse did not reveal differences between the genotypes, if one were to ignore the ANOVA and focus only on the point at 1.7 lux for the wildtypes, it appears that they masked better than the mutants at this illumination. We feel that this data point is more likely to be due to sampling error with a small group size ($n = 4$) in a steep part of the curve where variability is relatively high. It must be kept in mind that locomotor activity is not a simple reflex but a complex behavior that can be

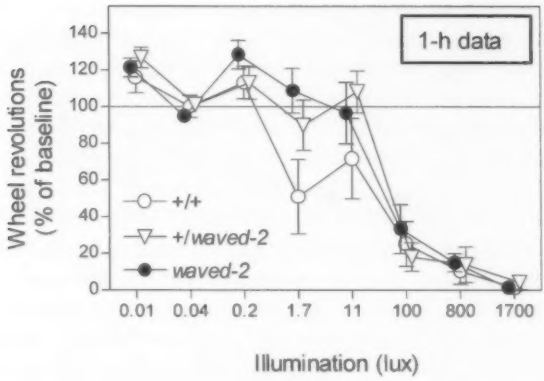

FIGURE 4 1 h pulses in Experiment 2. Means ± SEM of suppression of locomotion for the first 1 h of 3 h pulses of light of different illumination levels.

influenced by numerous factors, including disturbance, temperature, unknown "spontaneous" variations from day to day (even when conditions are kept constant), and hunger. *Egfr*^{wa2} homozygous mice are 10% lighter than heterozygous or wildtype mice; this depends partly on reduced food intake (Threadgill, unpublished observation). In order to use locomotion to measure masking, larger group sizes, in the order of 10, are required to give smoother curves (Mrosovsky, 2001).

In assessing the weight to be placed on this one point at 1.7 lux, it should be noted that the *Egfr*^{wa2} heterozygous mice did not show strong masking at this level, but were quite comparable to the homozygous mice. Also, on the

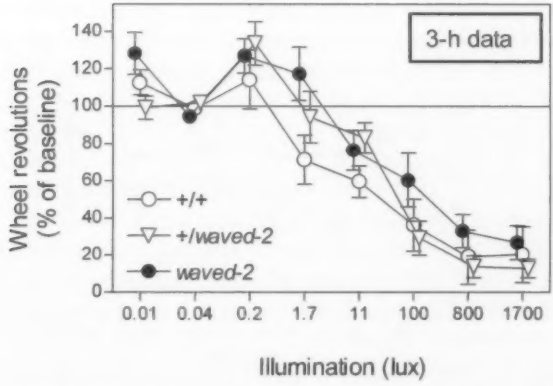

FIGURE 5 3 h pulses in Experiment 2. Means ± SEM of suppression of locomotion for full duration of 3 h pulses as a function of illumination level.

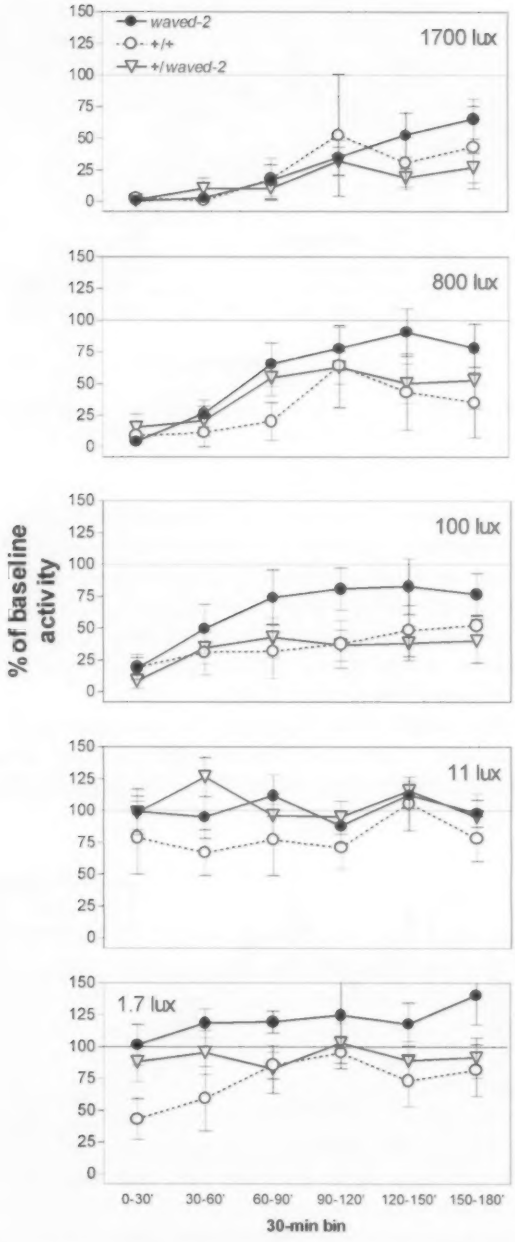

FIGURE 6 Time course of suppression of locomotion by 3 h light pulses in Experiment 2. Data for dimmer light pulses are not shown because there was little or no inhibition of activity.

ultradian cycle—a better, more extensive test than a single pulse in that it is based on comparing many periods of light and dark—there was no evidence that the $Egfr^{wa2}$ homozygous mice had impaired negative masking at 1.7 lux or other illuminations. The levels of light with the ultradian LD cycles were chosen to include masking scores around 75% activity in the dark, thus providing room for either worse (nearer 50% activity in the dark) or better masking (nearer 100% in the dark).

A second conceivable indication of worse masking in the $Egfr^{wa2}$ mutants concerned the time course of masking in the 3 h pulse in Experiment 2 at 11 lux. Although the masking scores did not differ among the genotypes, the interaction between genotype and time elapsed in the 3 h pulse did reach significance on this particular test, with the wildtypes tending to suppress more in the earlier times of the 3 h pulse.

From other experiments, it is known that melanopsin is important for the maintenance of negative masking over a long time period. Melanopsin knockout mice do not suppress activity for as long as wild-types (Mrosovsky and Hattar, 2003). So, it is conceivable that the $Egfr^{wa2}$ homozygous mutant mice have some deficit in melanopsin accounting for the data at 11 lux in Experiment 2. However, this is unlikely, because in Experiment 1 the time courses of activity suppression over the 3 h pulses were similar in $Egfr^{wa2}$ homozygous and heterozygous mice, and significant interactions between genotype and time within the 3 h pulse were not detected.

In Experiment 1, heterozygous rather than wildtypes provided a comparison group. Heterozygous $Egfr^{wa2}$ and wildtype mice are usually treated as equivalent (Roberts et al., 2002), and indeed in the 2001 study by Kramer and colleagues, the masking scores of their heterozygous and wild-type mice were very close, indicating that in the case of Experiment 1 the heterozygous mice provide valid controls equivalent to wild-types.

The fact is that, in our experiments, even without making allowance for the inherent variability of locomotion over a relatively short period, masking was quite similar in homozygous $Egfr^{wa2}$, heterozygous, and wild-type mice. Certainly, on the basis of our results with homozygous $Egfr^{wa2}$ mice alone, there is no support for the strong conclusions of Kramer and associates (2001) that EGFR signaling is "required" for negative masking. The Kramer and colleagues (2001) findings have been taken as identifying a common mechanism for clock control and masking control of locomotion. However, our data do not support a role for EGFR in masking. We do not consider this is the last word on this subject; nor do we see it as "the slaying of a beautiful hypothesis by an ugly fact" (Thomas Huxley). It is simply a statement to the effect that in our hands, with our procedures (which were extensive), we did not observe evidence linking EGFR dysfunction with deficits in masking.

ACKNOWLEDGMENTS

We thank Peggy Salmon, and Usha Ramanathan for help, and Franziska Wollnik for advice on statistics. Support came from the Canadian Institutes of Health Research (NM) and the National Institutes of Health (DWT).

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Chronobiology International, 22(6): 975–985, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500395094

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DIAZEPAM AFFECTS BOTH LEVEL AND AMPLITUDE OF RAT LOCOMOTOR ACTIVITY RHYTHM BUT HAS NO EFFECT ON CORE BODY TEMPERATURE

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The present study investigates the effects of a chronic administration of diazepam, a benzodiazepine widely used as an anxiolytic, on locomotor activity and body core temperature rhythms in male Wistar rats housed under 12 : 12 light : dark (LD) cycle conditions. Diazepam was administered subcutaneously for 3 wks in a dosage of 3 mg/kg body weight/day, 1 h before the onset of darkness. Diazepam increased the level of locomotor activity from the first day until the end of treatment, and also increased the amplitude of the activity circadian rhythm, but only on the third wk of treatment. Diazepam exerted no effects on the length of the period and did not affect the phase of the locomotor activity rhythm. The body temperature rhythm of rats was affected neither by short-term (a single injection) nor by long-term (every day for 3 wks) diazepam treatment. Diazepam lacked effect on body core temperature even on the first day of administration, thereby ruling out the possibility of drug tolerance development. The fact that diazepam affects locomotor activity, but not core body temperature, suggests that different mechanisms mediate the actions of diazepam on locomotor activity and on core body temperature.

Keywords Circadian rhythm, Diazepam, Locomotor activity, Body temperature, Wistar rat

INTRODUCTION

Disturbance of biological rhythms has been shown in many psychiatric disorders. Benzodiazepines are psychotropic drugs widely used in the treatment of anxiety, insomnia, and other disorders (Rickels et al., 2000). Diazepam, which can potentiate the action of the neurotransmitter

Submitted June 22, 2005, Returned for revision July 14, 2005, Accepted August 14, 2005
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γ-aminobutyric acid (GABA) activity through benzodiazepine receptors (Wakabayashi et al., 1991a), is known to produce phase-shifts of locomotor rhythms of hamsters (Houpt et al., 1986; Ralph and Menaker, 1986) and mice (Subramanian and Subbaraj, 1993) under constant conditions, to block light-induced phase advances in the locomotor activity rhythm (Ralph and Menaker, 1986), and to facilitate faster resynchronization of circadian rhythms to shifted light cycles (Childs and Redfern, 1981; Van Reeth and Turek, 1992). The observation that benzodiazepines, like melatonin, can induce a phase shift under constant conditions in hamsters and mice and can facilitate re-entrainment (Ralph and Menaker, 1986; Turek and Losee-Olson, 1987; Subramanian and Subbaraj, 1993; Yokota et al., 2000) suggests that treatment with benzodiazepines may also be capable of altering the entrainment pattern of rodents synchronized to the light : dark (LD) cycle. To test this hypothesis, experiments were conducted on the effects of a short-term (1 d) and long-term (21 d) diazepam administration on locomotor activity and body temperature rhythms under LD 12 : 12 conditions in Wistar rats.

MATERIALS AND METHODS

Animals and Experimental Procedures

Male (6-wk-old) Wistar rats were purchased from IFFA CREDO (L'Arbresle, France). The animals were housed in individual cages, with food and water available *ad libitum*, and maintained in a chronobiologic animal facility (Enceinte Autonome d'Animalerie, Ref. A 110-SP-6, ESI Flufrance, Arcueil, France). The chronobiologic facility was equipped with equidistant, sound-proof, temperature-controlled ($21 \pm 1.0^{\circ}C$) compartments each provided with independent LD cycles.

Surgical intervention was performed under aseptic conditions, 3 wks prior to commencement of drug treatment. Animals were lightly anesthetized by intraperitoneal administration of diazepam (Valium, Roche) (5 mg/kg) followed by ketamine (Ketalar, Parke-Davis) (80 mg/kg). Each animal was chronically instrumented with an intra-abdominal radiotelemetric implant (Model TA10TA-F40, Data Sciences Int., St. Paul, Mn, USA) to permit automatic recording of body temperature and general activity every 5 min throughout the 24 h using Dataquest III data acquisition software (Data Sciences Int.). During experimentation, radio waves from the implant were collected by a radar receiver RLA-1020 (Data Sciences Int.) placed beneath the cage. Animals were transferred to individual cages and synchronized to a reversed 12 : 12 LD regimen (light from 23 : 00 to 11 : 00 h) to allow dark-span sampling during the daytime work schedule of the investigators. During the first 5 d of recovery

following the surgical implantation of the sensor, rats were maintained under a normal 12L : 12D schedule.

From day 21, diazepam in a dosage of 3 mg/kg body weight/day was administered subcutaneously to rats ($n = 5$) daily, 1 h before lights off, for 3 wks. The dose of diazepam and time of drug administration were chosen according to previously published data (Wakabayashi et al., 1991b; Mailliet et al., 2001; Djeridane and Touitou, 2001). Control animals ($n = 5$) received only aqueous vehicle (Roche) equal in volume to the diazepam solution administered to experimental animals. Rats were returned to their home cages after injection. They were sacrificed by decapitation at the age of 12 wks, 7 h after the last injection (middle of the dark phase) and after 3 wks of treatment. All research procedures were performed in agreement with the principles of Laboratory Animal Care (NIH), French National Laws, and standards and ethics for animal biological rhythm research (Touitou et al., 2004).

Data Analysis

Chronos-Fit program (Zuther and Lemmer, Chronos-Fit, http://www.ma.uni-hedelberg.de/inst/phar/forschungLemmer.html, 2004) data analysis, an extended version of Win-ABPM-Fit (Zuther and Lemmer, 2003), was employed to elaborate actograms. With this program, actograms were constructed and double-plotted in a standard manner (Pittendrigh and Daan, 1976) to show the continuity of the rhythmic pattern of locomotor activity and body temperature. Data series were averaged over 30 min epochs for analysis. Methods used to analyze the temporal organization of locomotor activity and body temperature were fully described previously (Benstaali et al., 2002). Circadian rhythm analysis was performed by fitting of a cosine function (Witte et al., 2004) to the data using the Chronos-Fit program. The program fits a cosine curve to the measured data points, detects significant rhythmic changes (period lengths of 24, 12, 8, 6, 4.8, and 4 h), and derives estimates of the midline estimating statistic of rhythm (MESOR, *i.e.*, the rhythm-adjusted 24 h mean), amplitude (half of peak-to-trough of rhythmic change), and acrophase (peak time of each component cosine function) of the harmonics, together with the percent rhythm (an index of accounted for variance). Parameters of locomotor activity (quantified as counts/30 min) and body temperature (°C) across the 24 h were assessed by two-way analysis of variance (ANOVA), using group treatments and day of treatment as the two factors. One-way ANOVA followed by *post hoc* Dunnett's test was used to evaluate the effects of treatment on both parameters. In each analysis, differences between mean values were considered statistically significant if $p < 0.05$. Values are given as mean $\pm$ standard error of the mean (S.E.M.) for

MESOR and amplitude and as mean and the 95% confidence intervals for acrophase. The reference time of the acrophase is activity onset.

RESULTS

Under 12L:12D conditions, the subcutaneous administration of diazepam (3 mg/kg), 1 h before the onset of darkness, tends to increase the locomotor activity levels in drug-treated animals compared to vehicle-treated animals (Figure 1).

On day 1 of treatment, the mean level of locomotor activity was 39.43 ± 3.96 counts/30 min in diazepam-treated animals versus 26.16 ± 3.60 counts/30 min in vehicle-treated animals ($p < 0.05$). Nevertheless, the diazepam-induced increase in activity on day 1 did not reach significance. The amplitude of the 24 h pattern of locomotor activity rhythm averaged 14.57 ± 2.76 counts/30 min in diazepam-treated versus 14.59 ± 3.51 counts/30 min in vehicle-treated animals.

After 21 days of treatment, in contrast to day 1, diazepam significantly affected the 24 h pattern of locomotor activity. The level of locomotor activity increased to 49.20 ± 5.93 counts/30 min in diazepam-treated rats versus 25.64 ± 3.27 counts/30 min in vehicle-treated ones ($p < 0.01$). The amplitude averaged 27.02 ± 4.86 counts/30 min in diazepam-treated animals versus 12.51 ± 2.81 counts/30 min in vehicle-treated animals ($p < 0.05$).

The results of two-way ANOVA on locomotor activity function are presented in Table 1. The circadian amplitude of locomotor activity of the diazepam-treated rats was greater on day 21 than on days 1 ($p < 0.05$), 7 ($p < 0.01$), and 14 ($p < 0.05$). Moreover, the mean level of activity during the dark phase was significantly higher on day 21 (66.74 ± 5.21 counts/30 min) than on day 1 (49.56 ± 2.45 counts/30 min; $p < 0.01$), day 7 (50.08 ± 1.35 counts/30 min; $p < 0.01$), and day 14 (52.85 ± 2.29 counts/30 min; $p < 0.05$). No statistical significant differences in the mean level of these parameters (amplitude and MESOR) with diazepam treatment were observed between days 1, 7, and 14.

Diazepam did not modify the waveform or amplitude of the 24 h pattern of body temperature even after 3 wks of treatment (Figure 1). The 24 h mean (MESOR) of body temperature averaged 37.36 ± 0.014°C in diazepam-treated animals versus 37.32 ± 0.011°C in vehicle-treated animals (Table 2). During the dark phase (the activity phase of the animals), core body temperature averaged 37.67 ± 0.079°C in diazepam-treated animals versus 37.69 ± 0.041°C in vehicle-treated animals on day 1 of drug treatment, and 37.65 ± 0.087°C in diazepam-treated animals versus 37.64 ± 0.059°C in vehicle-treated animals on day 21 of drug treatment (Table 2).

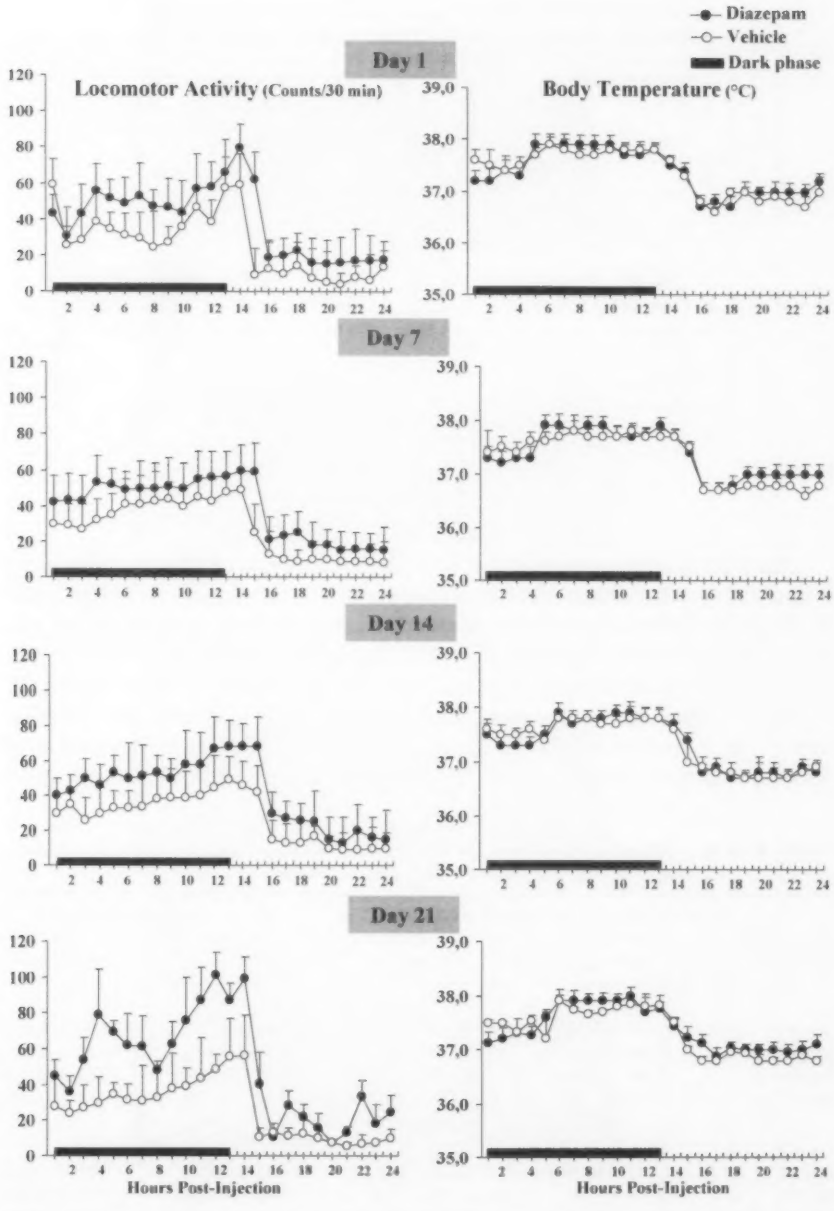

FIGURE 1 Time-course effect of diazepam (3 mg/kg) on locomotor activity and core body temperature in rats after a single subcutaneous injection of the drug on days 1, 7, 14, and 21. Diazepam was injected 1 h before the onset of darkness (lights off from 11:00 to 23:00 h). The black bar indicates the dark phase of the light:dark cycle. Each value is the mean ± S.E.M. of 5 rats.

TABLE 1 Two-way ANOVA Analysis on Locomotor Activity Function. Comparison Between Diazepam (DZP)-Treated and Control, and DZP-Treated Rats on Day 21 *vs.* days 1, 7, and 14

	24 h Mean levels		Amplitude	
	F	*P*	*F*	*P*
Days of DZP treatment *vs.* control				
Day 1	3.95	0.05	0.21	0.99
Day 7	3.62	0.05	0.14	0.95
Day 14	4.73	0.01	0.01	0.23
Day 21	4.80	0.01	4.64	0.05
DZP-treated day 21 *vs.* other days				
Day 1	0.06	0.20	3.60	0.05
Day 7	0.01	0.15	4.80	0.01
Day 14	0.07	0.32	4.20	0.05

Diazepam administration did not affect the phase of locomotor activity (Figure 2) and core body temperature (Figure 2) rhythms, even after 3 wks of drug administration. Furthermore, diazepam had no effects on the period length of the rhythms; both functions had a dominant 24 h periodicity. Minor (small-amplitude) 5.6 and 6 h periodicities were found by Cosinor analysis in locomotor activity and core body temperature, respectively. The parameters (MESOR, amplitude, acrophase, and minor and dominant period length) of the locomotor activity and body temperature

TABLE 2 24-Hour Mean Levels and Amplitude of Locomotor Activity and Core Body Temperature on Days 1, 7, 14, and 21 of Diazepam (DZP) Treatment. Means ± S.E.M.

Days of DZP treatment	Locomotor activity (counts/30 min)		Core body temperature (°C)	
	DZP-treated	Controls	DZP-treated	Controls
		24 h mean levels		
Day 1	39.43 ± 3.96*	26.16 ± 3.60	37.37 ± 0.086	37.35 ± 0.088
Day 7	39.06 ± 3.45*	27.46 ± 3.14	37.37 ± 0.086	37.30 ± 0.092
Day 14	42.08 ± 3.82**	27.71 ± 2.79	37.32 ± 0.094	37.31 ± 0.093
Day 21	49.20 ± 5.93**	25.64 ± 3.27	37.38 ± 0.079	37.31 ± 0.086
		Amplitude		
Day 1	14.57 ± 2.76	14.59 ± 3.51	0.41 ± 0.043	0.31 ± 0.035
Day 7	12.34 ± 1.45	12.50 ± 1.82	0.41 ± 0.031	0.33 ± 0.033
Day 14	13.84 ± 2.32	10.37 ± 1.59	0.43 ± 0.049	0.37 ± 0.037
Day 21	27.02 ± 4.86*/**	12.51 ± 2.81	0.39 ± 0.062	0.37 ± 0.048

*$p < 0.05$; **$p < 0.01$ *vs.* controls (ANOVA).

*$p < 0.05$ *vs.* controls and DZP-treated day 1 and day 14; **$p < 0.01$ *vs.* DZP-treated day 7 (ANOVA).

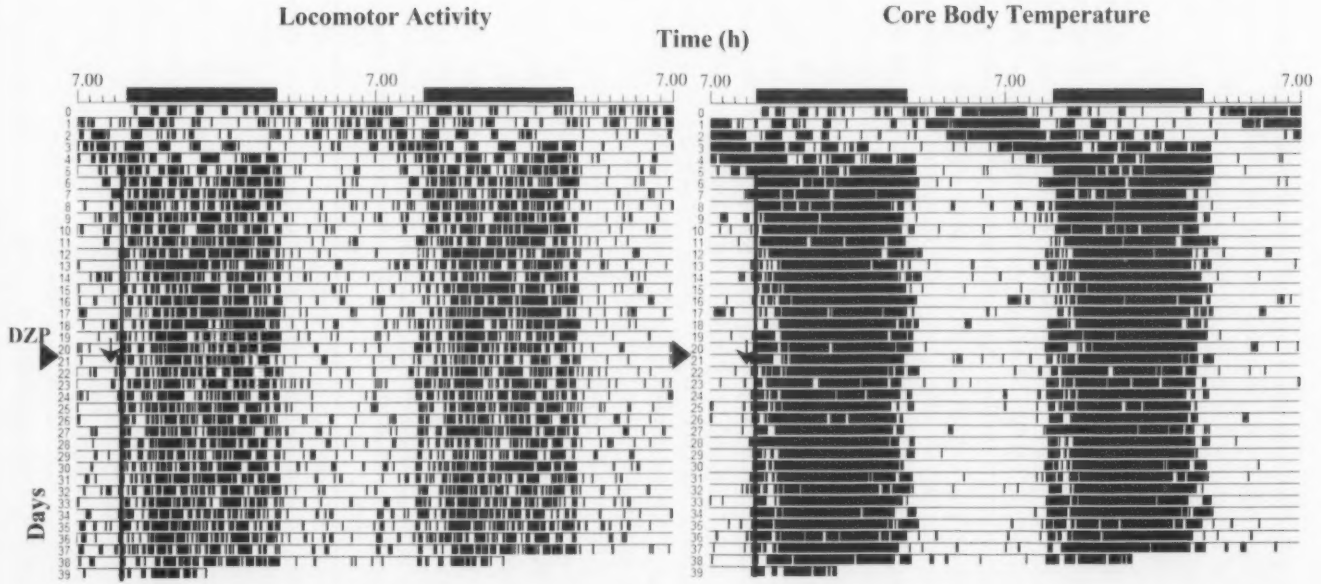

FIGURE 2 Double-plotted representation of locomotor activity and core body temperature in a single Wistar rat injected subcutaneously 1 h before the onset of darkness (lights off from 11:00 to 23:00 h) with diazepam (DZP) (3 mg/kg). Each horizontal line depicts the activity or temperature (black section in each line represents values above the 24 h mean, while the white section represents values below the mean) pattern over the 24 h span. Note that the two rhythms showed the same response to treatment. Arrows denote the time of DZP administration (10:00 h) and arrowheads the day of commencement of DZP treatment (day 21). During the first 5 d following surgery, rats were maintained under normal lighting schedule (lights on 11:00 to 23:00 h) for recovery. This figure also shows the adaptation time needed from the normal to inverse (L from 23:00 to 11:00 h: D from 11:00 to 23:00 h) lighting schedule. Days 6 to 20 were control days.

rhythms before (days 6 to 20) and after diazepam treatment (days 21 to 40) are summarized in Table 3.

DISCUSSION

The effects of diazepam (3 mg/kg) on body temperature and locomotor activity of Wistar rats under 12L : 12D conditions were examined by radiotelemetry. The pattern of body temperature was affected neither by an acute (1 d) nor by a chronic (3 wk) administration of diazepam in Wistar rats; the drug affected neither the circadian amplitude nor phase of the studied circadian functions. Martin and colleagues (1983) reported that diazepam has no effect on body temperature regulation, since no significant difference in body temperature occurred in rabbits receiving intramuscular diazepam (0.25 mg/kg) *vs.* vehicle treatment daily for 2 wks. Using radiotelemetry, Mailliet and colleagues (2001) previously showed that diazepam, administered intraperitoneally during light and dark periods, at the same dose we used (3 mg/kg), induced hypothermia in Wistar rats under 12L : 12D conditions. This discrepancy may be due to the experimental methods used (recording and monitoring) and/or mode, time, and duration of drug administration. Indeed, in our study diazepam was administered subcutaneouly as a single injection per day, while in the study by Mailliet and coworkers (2001) it was administered intraperitoneally, twice a day. In monkeys, diazepam has been shown to induce either hypothermia when administered peripherally by intramuscular injections, or hyperthermia when administered centrally into the lateral cerebral ventricule (Clark and Lipton, 1981).

TABLE 3 Parameters of Locomotor Activity and Core Body Temperature Rhythms Before (Days 6 to 20) and After Diazepam (DZP) Treatment (Days 21 to 40) Performed by Chronos-Fit Software. Means (± S.E.M. for MESOR, Amplitude, and Minor (M) and Dominant (D) Period Length; 95% Confidence Intervals for Acrophase)

	MESOR (counts/30 min)	Amplitude (counts/30 min) (°C)	Acrophase (h) (95% CI) (°C)	M period length (h)	D period length (h)
			Locomotor activity		
Before DZP treatment	40.46 ± 10.83	24.09 ± 6.67	17.49 (16.87–17.71)	4.6 ± 0.51	24
During DZP treatment	34.62 ± 9.58	26.31 ± 7.33	17.65 (17.09–17.84)	5.6 ± 0.245	24
			Core body temperature		
Before DZP treatment	37.41 ± 0.344	0.39 ± 0.0328	17.03 (16.72–17.14)	5.6 ± 0.245	24
During DZP treatment	37.31 ± 0.056	0.54 ± 0.0414	17.35 (16.87–17.52)	6	24

Locomotor activity levels were increased in diazepam-treated animals compared to vehicle-treated animals. A single subcutaneous administration of diazepam significantly affected the mean level but not amplitude of the 24 h pattern of locomotor activity. Diazepam increased the amplitude of the activity 24 h pattern during the third wk of treatment. Interestingly, in Sprague-Dawley rats, Sipos and associate (1999) showed that diazepam (3 mg/kg) administered intraperitoneally during the light period under 12L : 12D conditions did not increase measures of activity 23 h following drug administration, while in hamsters (*Mesocritus auratus*), Mrosovsky and Salmon (1990) showed that high doses of the benzodiazepine triazolam (>10 to 20 times more potent than diazepam) induced phase-shifts in wheel-running mediated through activity increases.

We also showed that diazepam failed to evoke changes in phase, even after 3 wks of drug administration. This is in good agreement with data on field mice *Mus booduga* subjected to a 12L : 12D regimen and offered the drug (0.25 mg/mL) through drinking water for 3 wks (Subramanian and Subbaraj, 1996). The suprachiasmatic nucleus (SCN) is known to be the major site of circadian rhythm generation (Inouye and Kawamura, 1982; Perreau-Lenz et al., 2004). The phase-shifting effect of diazepam on the circadian rhythm of locomotor activity in free-running hamsters is thought to be due to the direct effect of this drug within the SCN (Ralph and Menaker, 1986). The rhythmic nocturnal secretion of melatonin is dependent on the SCN. The rhythm-entraining properties of melatonin in rats are well established (Pitrosky et al., 1999). We have previously demonstrated that diazepam has an inhibitory effect on pineal melatonin synthesis in Wistar rats (Djeridane and Touitou, 2001, 2003a,b). Interestingly, it has been reported that the inhibitory effects of diazepam on pineal melatonin synthesis are not due to a direct effect of diazepam on the SCN (Wakabayashi et al., 1991b). Reciprocal effects of chronic diazepam and melatonin on brain melatonin and benzodiazepine binding sites have been demonstrated in rats (Atsmon et al., 1996). These data plus our present results–lack of effect of diazepam on the phase of the locomotor activity rhythm–suggest to us that the diazepam effects on the activity circadian rhythm of rats are not the result of a direct action on the SCN.

Administration of benzodiazepines is known to be associated with tolerance and a withdrawal syndrome on abrupt cessation (File, 1990). The lack of difference of core body temperature between day 1 and day 21 of diazepam treatment points out that this lack of effect of diazepam is not related to drug tolerance development due to the repeated administration of this benzodiazepine. One hypothesis raised to explain this lack of effect is that the dose of 3 mg/kg used in this study may be not pharmacologically sufficient to provoke maximal activation of the $GABA_A$ receptor. This hypothesis is corroborated by the fact that the 24 h mean level (MESOR)

of core body temperature was not changed on the first day of diazepam administration, which rules out the possibility of a tolerance development to repeated administration of diazepam. Another possibility is that the mechanisms mediating the action of diazepam on locomotor activity and on core body temperature are different.

In conclusion, this study shows that diazepam differentially affects core body temperature and locomotor activity; it increases the levels of locomotor activity from the first day until the end of treatment, and it increases the circadian amplitude of the locomotor activity rhythm during the third wk of treatment. However, no effect of diazepam treatment was observed on core body temperature. In contrast to studies showing that under free-running conditions, diazepam phase advances or phase delays the locomotor activity rhythm of hamsters (Martin and Menaker, 1986) and field mice *Mus booduga* (Subramanian and Subbaraj, 1993) and that it modulates the period (period-lengthening and period-shortening) of the locomotor activity rhythm of field mice *Mus booduga* (Subramanian and Subbaraj, 1996), we showed in the present study that under entraining 12L : 12D conditions, diazepam administered 1 h before the dark span does not alter the phase nor the period of the locomotor activity circadian rhythm of Wistar rats. Further investigations are needed to explain the precise mechanisms of action of diazepam on core body temperature and motor activity and their circadian rhythms.

ACKNOWLEDGMENTS

The authors are deeply indebted to Roche labs for their generous gift of diazepam and the vehicle solution.

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Chronobiology International, 22(6): 987–996, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500395110

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DOSING-TIME–DEPENDENT DIFFERENCES IN LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY IN RATS

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Dosing-time–dependent differences in lipopolysaccharide (LPS)-induced liver injury were examined in rats housed under a 12 h light : dark (LD) cycle. LPS (5 mg/kg) was intravenously injected into different groups of rats at 2, 14, or 20 h after light on (HALO). Elevations in serum liver enzymes after 14 HALO were significantly greater than those after 2 HALO. These parameters were lower in rats given LPS at 20 HALO, compared to 14 HALO. The number of polymorphonuclear cells (PMN) in the liver and the amount of hepatic myeloperoxidase activity, which reflects the number of PMN in liver tissues, was significantly greater in the 14 than in the 2 HALO group. In addition, hepatic interleukin-6 (IL-6) production in the 14 HALO group was enhanced compared to that in the 2 HALO trial. These results suggest that LPS-induced liver injury is greater during the early active than during the early resting period. Dosing-time–dependent variation in the accumulation of PMN in the liver and, potentially, subsequent IL-6 production in liver tissues might be involved in this phenomenon.

Keywords Chronotoxicity, Circadian rhythm, Lipopolysaccharide, Liver toxicity, Polymorphonuclear cells, Interleukin-6

INTRODUCTION

Lipopolysaccharide (LPS) is a fragment of the cell wall of gram-negative bacteria. Systemic injection of LPS causes release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Bluthe et al., 2000; Tollner et al., 2000), that signal the central nervous system and induce signs of illness, such as elevated body temperature (fever), reduced locomotor activity, and altered sleep-wake behavior

Submitted September 3, 2005, Returned for revision September 13, 2005, Accepted September 15, 2005

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(Luker et al., 2000; Mathias et al., 2000; Franklin et al., 2003; Marpegan et al., 2005). LPS also induces liver injury through the enhanced production of cytokines, including TNF-α and IL-6 (Luster et al., 1994; Tsuji et al., 1999; Lawson et al., 2000).

Recent advances in chronopharmacology demonstrate that the toxicities of many chemical and non-chemical agents vary in severity according to the biological time of their administration (Cambar et al., 1997; Dridi et al., 2005; Cui et al., 2004, 2005; Daniel and Touitou, 2004; Filipski et al., 2004; Rebuelto et al., 2004; Rich et al., 2002). The chronotoxicity of the LPS-induced illness has been extensively examined in both laboratory animals and humans (Pollmacher et al., 1996; Luker et al., 2000; Mathias et al., 2000; Franklin et al., 2003; Marpegan et al., 2005).

This dosing-time–dependent differences in LPS-induced liver injury has not been previously explored. Thus, the present study was undertaken to examine whether the severity of LPS-induced liver injury is influenced by the circadian time of administration, and if so, to evaluate the potential mechanisms.

MATERIALS AND METHODS

Animals

Male Wistar rats (9 to 10 wks old; weight, 195 to 225 g; SLC Ltd., Shizuoka, Japan) were maintained in a specific pathogen-free environment with unrestricted access to water and a standard diet under a 12 h light : dark (LD) cycle (07:00 h light on and 19:00 h light off) in the animal center of the Jichi Medical School. The study was performed in accordance with the Jichi Medical School guide for laboratory animals and ethical principles and standards for the conduct of human and animal biological rhythm research advocated by the Journal (Touitou et al., 2004).

Chemical

LPS (*Escherichia coli* O55:B5; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline at 5 mg/mL and kept at -20°C until use in each experiment.

Experiment 1: Dosing-Time–Dependent Differences in LPS-Induced Liver Injury

To determine the dosing-time–dependent differences in liver injury, LPS (5 mg/kg) or vehicle alone was injected through the penile vein in different but comparable groups of rats at 2, 14, or 20 h after light on (HALO). Blood samples were obtained just before and at 1, 3, 6, and 12 h after the LPS or vehicle injection at each circadian time.

Because Experiment 1 showed that the LPS-induced liver injury was significantly greater in the 14 than in the 2 HALO group, subsequent experiments were performed at 2 and 14 HALO.

Experiment 2: Dosing-Time–Dependent Differences in LPS-Induced Elevations in Serum Cytokines

In the preliminary study, LPS (5 mg/kg) was injected in rats, and blood samples for the measurement of cytokines were obtained at 1, 3, and 6 h after the timed injection. Because serum concentrations of TNF-α and IL-6 showed their peaks at 1 and 3 h (data not shown), respectively, blood samples were obtained at 1 h for TNF-α and at 3 h for IL-6 after the LPS injection at 2 or 14 HALO.

Experiment 3: Dosing-Time–Dependent Change in LPS-Induced Cytokine Production in Liver Tissues (Tissue Culture Study)

In the preliminary study, the appropriate dose of LPS and timing of sampling were determined. Because the viability of liver tissues after the injection of 5 mg/kg LPS was remarkably low, the dosage of 1 mg/kg LPS was used for the experiment. LPS (1 mg/kg) was injected into animals, which were sacrificed 1 h later. TNF-α and IL-6 were determined in the supernatant of liver tissue samples at 2 and 6 h after the incubation of liver slices. The study showed that TNF-α was significantly elevated after 2 h of incubation, but was not further elevated at 6 h, while IL-6 was not elevated after 2 h of incubation, but was significantly elevated after 6 h of incubation (data not shown). Therefore, cytokine concentrations in the supernatant were determined after 6 h of incubation in the subsequent experiments.

Rats received LPS (1 mg/kg) at 2 or 14 HALO and were sacrificed 1 h later. Cytokine production in liver tissues was determined as previously reported (Luster et al., 1994). Liver slices measuring 6 mm in diameter, using a cylindrical core with a sharpened length of metal tubing, were obtained from the central right hepatic lobule. The 200 mm sections (9.5 to 10.5 mg wet weight) were cut with a Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL, USA) and collected in cold Hank's balanced saline solution. Each slice (2 slices per rat) was incubated in 12 mm Transwell culture dishes (3.0 μm pore; Corning Costar Corp., Cambridge, MA, USA) containing 500 μL of RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% FCS (Sigma-Aldrich). Culture dishes were filled with 100% O_2 for 1 min, and thereafter, at 37°C for up to 6 h. After the first 30 min of incubation, culture medium was replaced with fresh medium. Supernatants were obtained from tissue culture medium 6 h after the initiation of incubation.

Experiment 4: Dosing-Time–Dependent Differences in Tissue Myeloperoxidase (MPO) Activity and Polymorphonuclear Cell (PMN) Infiltration in Liver Tissue

LPS (5 mg/kg) was injected at 2 or 14 HALO, and 12 h later rats were sacrificed to obtain the damaged liver tissue. Livers were immediately frozen and stored at −70°C until analysis.

Measurements of Transaminase and Cytokines

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by an ultraviolet method using an auto-analyzer (Hitachi 7170, Japan). Serum and medium concentrations of TNF-α and IL-6 were determined using sandwich enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Inc., Camarillo, CA, USA).

Measurement of MPO Activity in Liver Tissues

Tissue samples were homogenized in 10 ml phosphate-buffered saline and centrifuged at 100,000 G under 4°C for 30 min. The MPO activity was measured using a modified method of Shindler and colleagues (1997). A reaction mixture containing the supernatant (10 μL) and ABTS (100 μM, Roche, Japan) with H_2O_2 was incubated for 60 min at 25°C in a 96-well plate. The optical density at 414 nm was determined by a plate reader (SPECTRAmax 340, Molecular Devices, Sunnyvale, CA, USA). MPO activity was calculated by the use of an extinction coefficient 3.6×10^4/mL·cm for ABTS and normalized to the protein level (μmol/mg protein).

Measurement of PMN Number in Liver Tissues

More than two sections were prepared from each liver sample and then stained with hematoxilin-eosin. The PMN were morphologically identified and counted under 400-fold magnification of a light microscope. All fields were examined by two pathologists using a blind design. Data were expressed as PMN/50 high-power field (HPF).

Statistical Analysis

Data are shown as the mean ± SD. Differences in mean values were tested using analysis of variance (ANOVA) and Student's *t*-test, with statistical significance taken as $p < 0.05$.

RESULTS

Serum concentrations of AST and ALT rose gradually for up to 12 h following LPS injection. There were no significant differences in these parameters between the three HALO injection times of 1, 3, and 6 h following treatment (data not shown). At 12 h, the mean serum concentrations of AST and ALT following LPS injection during the early active period (14 HALO) were significantly ($p < 0.05$) greater than they were following LPS injection during the early resting period (2 HALO) (Figure 1). The concentration of these enzymes following LPS injection during the late active period (20 HALO) was slightly lower than that after LPS injection at 14 HALO (Figure 1).

The serum concentrations of IL-6 and TNF-α rose following LPS injection (Figure 2). The elevations were significantly ($p < 0.05$) greater following injection at 14 than 2 HALO. IL-6 production in liver tissue was also greater following the LPS injection at 14 HALO ($p < 0.05$); however, no such dosing-time–dependent difference was detected in TNF-α production (Figure 3).

The number of PMN ($p < 0.05$) and the amount of MPO activity ($p < 0.01$) in the liver tissue were significantly greater following the 14 than the 2 HALO injections of LPS (Figure 4).

DISCUSSION

Previous studies have examined certain aspects of the chronotoxicity of LPS in nocturnally actively rodents. For example, motor activity of rats was inhibited after injection of LPS at 15 HALO (early active period), but not at 3 HALO (early resting period) (Luker et al., 2000). LPS-induced phase-shift of the circadian rhythm of motor activity was also detected at 15 HALO, but not at 3 HALO, in mice (Marpegan et al., 2005). On the other hand, elevation in the low-frequency components (1.5 to 7 Hz) of the electroencephalogram after LPS injection at 0 HALO (beginning of resting period) was significantly greater than after LPS injection at 12 HALO (beginning of active period) in rats (Mathias et al., 2000). In addition, it is reported that elevation in body temperature following injection of LPS at 3 and 15 HALO did not differ significantly in rats (Sugimoto et al., 1996; Luker et al., 2000). The present study showed that LPS-induced liver injury is dependent on the circadian time of LPS injection, and the extent of liver injury is greater after injection at 14 HALO (early active period) than at 2 HALO (early resting period). Thus, choronotoxic profiles of LPS depend on the variables that are influenced by it.

Endotoxin-induced liver injury is the result of multiple factors, in which PMN are involved (Doi et al., 1993; Chosay et al., 1997). In the present study, the number of PMN in the liver and hepatic MPO

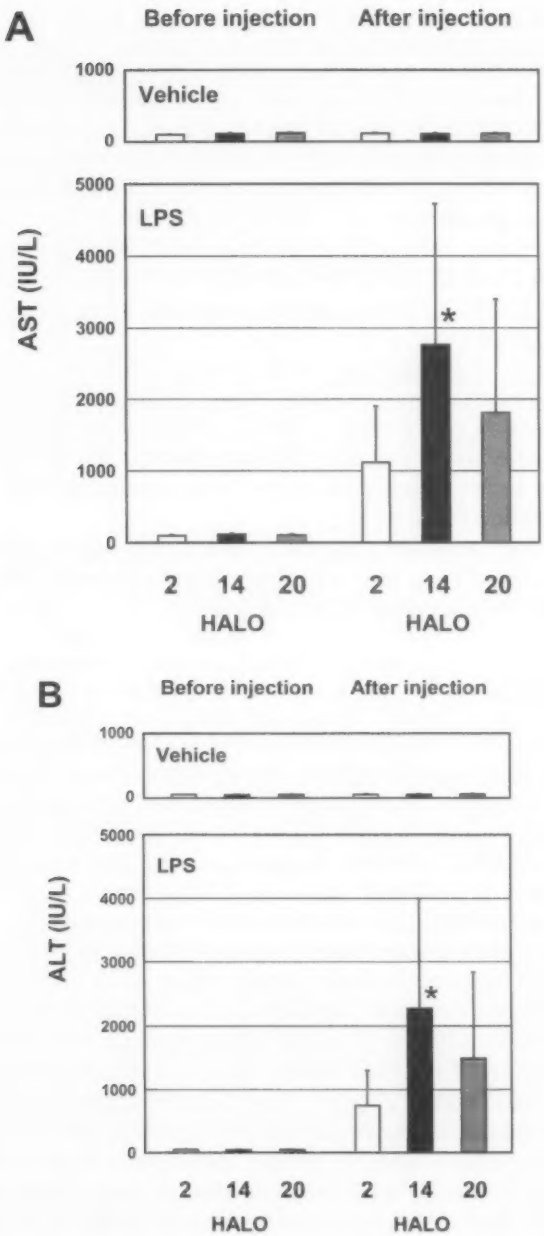

FIGURE 1 Dosing-time–dependent differences in serum AST (A) and ALT (B) 12 h after LPS injection at 2, 14, or 20 HALO in rats. Data are means ± SD, $n = 5$ in each vehicle-treated group, and $n = 10$ in each LPS-treated group. $^*p < 0.05$ *vs.* 2 HALO.

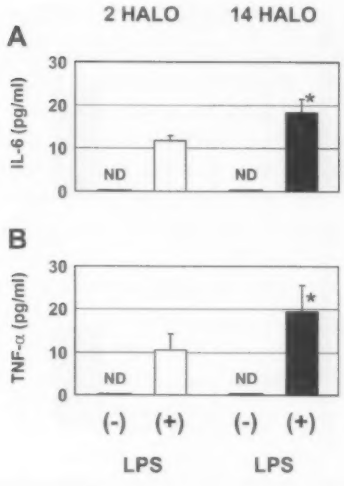

FIGURE 2 Dosing-time–dependent differences in serum IL-6 (A) and TNF-α (B) after LPS injection at 2 or 14 HALO in rats. Blood samples were obtained at 1 and 3 h after injection for measurement of TNF-α and IL-6, respectively. These cytokines were not detected (ND) after vehicle injection [LPS(−)]. Data are means ± SD, $n = 5$ in each group. $^*p < 0.05$ *vs.* 2 HALO.

content, which reflects the number of PMN in the liver, was significantly greater after LPS dosing at 14 HALO than at 2 HALO. This finding indicates that the LPS-induced accumulation of PMN in liver tissues is dependent on the circadian time of treatment, which in part gives rise to

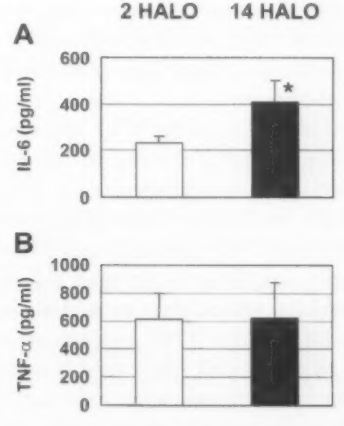

FIGURE 3 Effects of dosing-time on IL-6 (A) and TNF-α (B) concentrations in medium after incubation of liver slices of rats treated with LPS at 2 or 14 HALO. Data are means ± SD, $n = 5$ in each group. $^*p < 0.05$ *vs.* 2 HALO.

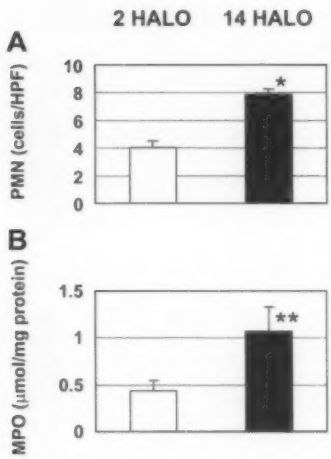

FIGURE 4 Number of polymorphonuclear cells (PMN) (A) and myeloperoxidase (MPO) activity (B) in liver tissues 12 h after LPS injection timed at 2 or 14 HALO in rats. Data are means ± SD, $n = 5$ in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* 2 HALO.

the dosing-time–dependent differences in the severity of liver injury as observed in this study. The recruitment of PMN into the liver requires several families of adhesion molecules. The initial contact of PMN with the endothelium is mediated by selectins and their ligands, which induce the rolling of PMN along the vessel wall (Granger and Kubes, 1994). This rolling phenomenon is a prerequisite for the subsequent firm adhesion and transmigration that is mediated by members of the integrin family (Smith, 1992). Based on the present data, it is speculated that the influences of LPS on adhesion molecules, such as the selectins and integrin family, vary according to the circadian time of its dosing. Additional studies are needed to address the issue.

Proinflammatory cytokines, such as TNF-α and IL-6, are produced in response to LPS and are thought to mediate many of its related effects (Tracey and Lowry, 1990). Many cell types contribute to the inflammatory cytokine response to LPS, including PMN (Ohira et al., 1995; Nick et al. 1999). In the present study, the serum concentration of IL-6 and its hepatic production were significantly greater following LPS injection at 14 compared with 2 HALO. Although serum TNF-α was also greater following the 14 HALO trial, hepatic TNF-α production of the two trials did not differ significantly. It is generally accepted it is the local concentrations of cytokines in tissues, rather than serum levels, that more likely reflect the biological response. Therefore, we think that the dosing-time–dependent differences in LPS-induced IL-6 production in liver tissues are involved in the mechanism of LPS chronotoxicity.

Glutathione (γ-glutamylcysteinylglycine) is a ubiquitous tripeptide thiol and an integral part of the endogenous cellular antioxidant defense system. For example, glutathione has been shown to play an important role in cellular defenses against LPS-induced liver injury in mice (Uedono et al., 1997). Previous studies demonstrated that hepatic glutathione content is greater at 2 than at 14 HALO in untreated mice (Neuschwander-Tetri and Rozin, 1996; Li et al., 1997). Therefore, circadian variation of hepatic glutathione might also contribute to the dosing-time–dependent differences in LPS-induced liver injury.

In summary, the present study showed that LPS-induced liver injury is greater after dosing during the early active than during the early resting period. Dosing-time–dependent differences in the accumulation of PMN, and potentially subsequent IL-6 production, in the liver tissues might be involved in this phenomenon.

ACKNOWLEDGMENTS

The authors greatly thank Prof. Fujio Kayama and Atsuko Kikuchi (Department of Health Science, Jichi Medical School) for technical advice of organ slice system, and Dr. Koichi Mizuta (Department of Pediatric Surgery, Jichi Medical School) for pathological study.

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Chronobiology International, 22(6): 997–1011, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500397892

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TIME-OF-DAY EFFECTS ON MYOELECTRIC AND MECHANICAL PROPERTIES OF MUSCLE DURING MAXIMAL AND PROLONGED ISOKINETIC EXERCISE

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The aim of this study was to examine the time-of-day (TOD) effects in myoelectric and mechanical properties of muscle during a maximal and prolonged isokinetic exercise. Twelve male subjects were asked to perform 50 maximal voluntary contractions (MVC) of the knee extensor muscles at a constant angular velocity of $2.09\,rad \cdot sec^{-1}$, at 06:00 and 18:00 h. Torque and electromyographic (EMG) parameters were recorded for each contraction, and the ratio between these values was calculated to evaluate variations of the neuromuscular efficiency (NME) with fatigue and with TOD. The results indicated that maximal torque values ($T_{45}Max$) was significantly higher (7.73%) in the evening than in the morning ($p < 0.003$). The diurnal variation in torque decrease was used to define two phases. During the first phase (1st to the 26th repetition), torque values decreased fast and values were higher in the evening than in the morning, and during the second phase (27th to the 50th repetition), torque decreased slightly and reached a floor value that appeared constant with TOD. The EMG parameters (Root Mean Square; RMS) were modified with fatigue, but were not TOD dependent. The NME decrease–significantly with fatigue, showing that peripheral factors were mainly involved in the torque decrease. Furthermore, NME decrease was greater at 18:00 than at 06:00 h for the vastus medialis ($p < 0.05$) and the vastus lateralis muscles ($p < 0.002$), and this occurred during the first fatigue phase of the exercise. In conclusion, the diurnal variation of the muscle fatigue observed during a maximal and prolonged isokinetic exercise seems to reflect on the muscle, with a greater contractile capacity but a higher fatigability in the evening compared to the morning.

Keywords Diurnal variation, Torque, Electromyography, Muscle fatigue, Isokinetic exercise

Submitted May 25, 2005, Returned for revision June 13, 2005, Accepted August 11, 2005
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INTRODUCTION

Circadian fluctuations have been observed under experimental conditions in many biological variables that induce time-of-day (TOD) modifications in response to exercise (Shephard, 1984; Cable and Reilly, 1987; Reilly and Brooks, 1990; Melhim, 1993; Gauthier et al., 1996). Previous studies showed that maximal force (Gauthier et al., 2001), anaerobic power and capacity (Melhim, 1993; Souissi et al., 2002), and aerobic performances (Atkinson and Reilly, 1996; Atkinson et al., 2005) are TOD-dependent. It appeared that muscle contractile properties have a circadian variation for short-term exercise, but whether this rhythmicity is maintained when exercise is prolonged has yet to be explored. The circadian rhythm of muscular voluntary strength has now been clearly demonstrated among all determinants of athletic performance, (Reinberg et al., 1988; Gauthier et al., 2001). This rhythm is characterized by an acrophase (time of the maximal level of the rhythm) between 17 : 00 and 19 : 00 h, a bathyphase (time of the minimum) between 04:00 and 06 : 00 h, and an amplitude ranging between 3% (Atkinson et al., 1993) to 21.2% (Coldwells et al., 1994), depending on the population, groups of muscles studied, and experimental conditions.

The capacity of athletes to maintain a high level of force, while an essential factor in performance, is limited by the phenomenon of fatigue. Muscle fatigue refers to the decrease in the ability to produce and/or maintain a strength level (Stephenson et al., 1998). During sustained maximal voluntary contractions, a continuous decrease in strength representative of fatigue is generally observed (Lunde et al., 1998; Perry-Rana et al., 2002). Factors involved in muscle fatigue have been explored extensively. However, the assignment of the fatigue phenomenon to central and/or peripheral origin has not been clearly defined (Bigland-Ritchie et al., 1986; Duchateau and Hainaut, 1986; Lunde et al., 1998). Taking this fact into consideration, the non-invasive technique of surface electromyography (EMG) can provide significant objective criteria for evaluating the origin of muscle fatigue (Kamen and Caldwell, 1996). In fact, the calculated ratio between strength values and corresponding EMG values are used to evaluate neuromuscular efficiency (NME), which is a component of neuromuscular fatigue, and NME variations are used to describe peripheral and/or central modifications which occur during muscle fatigue (Bigland-Ritchie, 1986). The changes in the NME point to two types of fatigue: a decrease of the NME is classified as "peripheral fatigue" and a constant NME associated with force decrease is classified as "central fatigue" (Bigland-Ritchie et al., 1978). It has been suggested that muscle properties are TOD-dependent during maximal and brief exercise; however, yet to be studied is whether or not such diurnal variation is apparent when exercise is prolonged. The aim of this study was to

evaluate, during a maximal and prolonged exercise performed under isokinetic conditions, the TOD effects on mechanical and myoelectric properties of muscle.

MATERIAL AND METHODS

Subjects

Twelve diurnally active male subjects (age, 24.0 ± 0.7 yrs; body mass, 76.0 ± 2.1 kg; height, 182.2 ± 1.6 cm) participated in the investigation. This study was granted ethical approval by the ethics committee (CHU Cote de nacre, Caen, France) and complied with the requirements of the Journal for the conduct of biological rhythm research on human beings (Touitou et al., 2004). The subjects were all volunteers and gave their informed consent after receiving a detailed explanation of the study protocol. They were categorized either as "moderately morning type" ($n = 4$) or as "neither type" ($n = 8$), on the basis of their answers to the Horne and Östberg (1976) self-assessment questionnaire, which assesses morningness-eveningness.

Experimental Procedure

In order to evaluate the diurnal fluctuation in muscle contractile properties, two test sessions (TS) were conducted (at 06:00 and 18:00 h) corresponding, respectively, to the minimum and the maximum daytime levels of force for the same experimental population as that of Gauthier and colleagues (2001). Each subject performed both TS, the timing of which was randomized. Each test session was separate by a 36 h rest period. Instructions about sleep and diet were given to the subjects prior to the experimentation. The subjects were asked to keep as closely as possible to their usual sleeping habits with a minimum of 6 h sleep taken on the night proceeding each test session. During the period of investigation, subjects were prohibited from consuming food, beverages, or any known stimuli (*e.g.*, caffeine) or depressants (*e.g.*, alcohol) that would possibly enhance or compromise alertness. Subjects were requested to avoid strenuous activity during the 24 h proceeding each test session. Subjects that were tested at 06:00 h were requested to come to the laboratory at 05:00 h, having eaten nothing. Only one glass (15 to 20 cl) of water was allowed, to avoid the effects of postprandial thermogenesis. Throughout the protocol period, the laboratory temperature was kept constant (19.9 ± 0.2°C) to minimize the effects of temperature variations on muscle function (Bergh and Ekblom, 1979).

Data Collection

The week before the experiment, all subjects performed one session of familiarization to the protocol and measurement apparatus. Each test session started with 20 min of rest, during which subjects remained supine. At the end of this rest period, oral temperature (Omron®, accuracy: 0.05°C) was recorded.

Instantaneous muscular torque was recorded using an isokinetic dynamometer Cybex II type (CYBEX® International, Belton, USA). The subject was seated on an arm chair and his thigh was held with a belt. This ankle was attached to the arm of the ergometer with a belt. The subject was positioned so that the rotational axis of the knee joint was aligned with the motor-axle of the ergometer. In this position, after three trials of three maximal familiarization repetitions (performed at 1.05, 2.09, and 3.14 $rad \cdot sec^{-1}$), subjects performed 50 concentric maximal voluntary contraction (MVC) of the dominant leg extensors, at an imposed angular velocity of 2.09 $rad \cdot sec^{-1}$. The range of motion was standardized from 0 to 90° of knee flexion (0° corresponding to the full extension of the leg). Flexion was carried out passively at a constant angular velocity of 2.09 $rad \cdot sec^{-1}$. Total time of one movement (active extension/passive flexion) was 1.5 sec.

The surface EMG was recorded from the vastus medialis (VM), vastus lateralis (VL), rectus femoris (RF), and biceps femoris (BF) muscles. Pre-amplified surface silver-silver chloride electrodes with an inter-electrodes distance of 35 mm (BIOPAC Systems®, Santa Barbara, California, USA) were put in place after the skin was shaved and cleaned with alcohol-ether. The electrodes were positioned over the belly of each studied muscle, arranged in parallel to the muscle fibers. The position of the electrodes was marked with ink dots to ensure standardized measurements between the two sessions. The amplification of the EMG signals was made with a bandwidth of 12 to 500 Hz (Common Mode Rejection Ratio = 95 dB; Z input = 100 MΩ; gain = 350) and a sampling rate of 1000 Hz.

Data Analysis

Torque and the corresponding EMG activity were recorded for each of the 50 movements performed by the subjects during each test session. The torque value was measured at a constant angle of 45° (T_{45}) (0° corresponding to the full extension of the leg). During each test session and for each subject, maximal T_{45} value ($T_{45}Max$) was recorded among the 50 movements to determine the maximal torque level. Next, to evaluate the fatigue during exercise, T_{45} values were averaged by samples of two consecutive repetitions. The muscles' electrical activity was analyzed by the

AcqKnowledge data analysis software (Model MP100A; BIOPAC Systems®, Santa Barbara, California, USA) and assessed by measuring the Root Mean Square (RMS) values. The RMS were calculated for each repetition for a time period corresponding to a 40° range of motion This standardized 40° range of motion corresponded to 65 to 25° knee extension. For each subject and each test session, the calculated RMS values were normalized (RMS_{norm}) to the highest value found for the 50 muscle contractions, and were next averaged by samples of two consecutive repetitions. Neuromuscular efficiency (NME) was calculated for the VM, VL, and RF muscles as the ratio between the torque and corresponding RMS_{norm} values.

Statistical Analysis

Statistical analysis was performed using Statistica® (Statsoft, Tulsa, Oklahoma, USA). A paired Student's *t*-test was used to identify diurnal fluctuations in oral temperature and T_{45}Max. In order to identify the fatigue and to shed light upon its diurnal variation, the values of T_{45}, RMS_{norm}, and NME were subjected to a two-way analysis of variance (ANOVA) for repeated measures (TOD × Fatigue). A Least Significant Different (LSD) Fisher test was used for *post hoc* analysis when significant effects were found. Values are mean (±SE). In all tests, $p < 0.05$ was indicative of statistical significance.

RESULTS

Temperature

A significant diurnal variation was found for the at-rest oral temperature ($F_{(1-11)} = 27.59$; $p < 0.001$). The mean value of oral temperature measured at 18 : 00 h (36.51 ± 0.07°C) was higher than the one measured at 06 : 00 h (35.63 ± 0.15°C), and the amplitude [(temperature at 18 : 00 h − temperature at 06 : 00 h)/2] of the rhythm was 0.88 ± 0.17°C.

Maximal Torque

There was evidence of diurnal fluctuation in T_{45}Max developed ($F_{(1,11)} = 14.39$; $p < 0.003$). Mean values significantly increased from 155.8 ± 5.3 N.m. at 06 : 00 h to 169.5 ± 6.3 N.m. at 18 : 00 h, an increase of 7.73 ± 1.9%.

Torque Decrease

Application of ANOVA to the data obtained throughout the exercise showed first a fatigue effect ($F_{(24,264)} = 258.59$; $p < 0.001$), with a

significant T_{45} values decrease through the 50 repetitions of both sessions. This decrease amounted to 63.3 ± 2.0% and 65.6 ± 1.7% at 06 : 00 h and 18 : 00 h, respectively (Figure 1). Second, no TOD effect ($F_{(1,11)} = 3.57$; $p = 0.08$) was found in the T_{45} values recorded at 18 : 00 h and at 06 : 00 h. Third, a significant interaction between fatigue and TOD ($F_{(24,264)} = 2.48$; $p < 0.001$) was detected in T_{45} values decrease, with the effect being more prominent in the evening than morning. *Post hoc* LSD analysis showed that the diurnal fluctuation of the T_{45} decrease was divided in two parts: a first part, when significant differences in the morning/evening tests were only observed from the 1st to the 10th, 17th to the 22nd, and 25th to 26th repetitions, and a second part, after the 26th repetition, when no significant difference between 06 : 00 h and 18 : 00 h was observed (Figure 1).

Electromyographic Parameters

The statistical analysis showed that during 50 MVC at 2.09 rad · s^{-1}, the RMS_{norm} values were significantly modified with fatigue for the VM ($F_{(24,264)} = 2.93$; $p < 0.001$), VL ($F_{(24,264)} = 5.83$; $p < 0.001$), and RF ($F_{(24,264)} = 5.84$; $p < 0.001$) muscles. The mean decrease recorded between the highest first and the last lowest values ranged from 17.1 ± 3.5% to 30.1 ± 4.2%, depending on the muscle and TOD (Table 1). The analysis showed that the RMS_{norm} values recorded throughout exercise in VM ($F_{(24,264)} = 0.61$; $p = 0.92$), VL ($F_{(24,264)} = 0.87$;

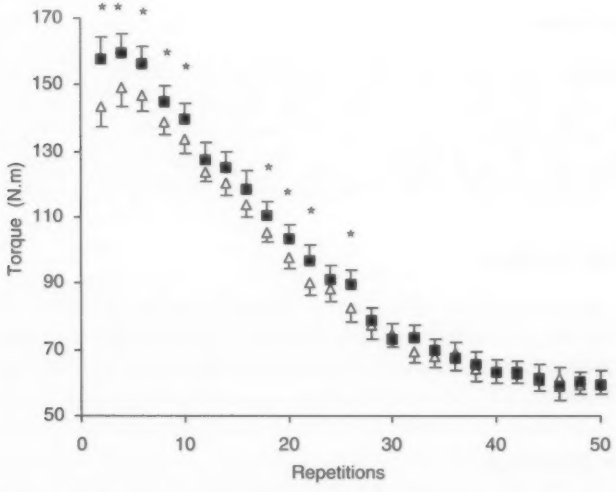

FIGURE 1 Relationship between Torque values and repetition number at 06 : 00 (△) and 18 : 00 h (■). Values are mean ± SE. ($n = 12$). *Indicates significant difference between 06 : 00 and 18 : 00 h.

TABLE 1 Variation (%) of RMS_{norm} and NME Values for Vastus Medialis (VM), Vastus Lateralis (VL), Rectus Femoris (RF), and Biceps Femoris (BF) muscles during a 50 MVC bout at 06:00 and 18:00 h

Variable	Muscle	06 : 00 h	18 : 00 h	Difference: evening/morning
RMS_{norm}	VM	-20.7 ± 4.8^a	-17.1 ± 3.5^a	$F_{(24,264)} = 0.61$; $p = 0.92$
	VL	-30.1 ± 4.2^a	-24.9 ± 5.1^a	$F_{(24,264)} = 0.87$; $p = 0.64$
	RF	-27.6 ± 6.0^a	-29.6 ± 3.5^a	$F_{(24,264)} = 0.36$; $p = 0.99$
	BF	$+37.9 \pm 3.7^a$	$+38.3 \pm 1.8^a$	$F_{(24,264)} = 0.66$; $p = 0.88$
NME	VM	-67.3 ± 2.1^a	-70.7 ± 1.7^a	$F_{(24,264)} = 1.60$; $p < 0.05^a$
	VL	-62.6 ± 2.1^a	-66.4 ± 2.1^a	$F_{(24,264)} = 2.21$; $p < 0.002^a$
	RF	-58.5 ± 4.1^a	-63.6 ± 3.5^a	$F_{(24,264)} = 0.68$; $p = 0.87$

Values are means $\pm$ SE ($n = 12$).
[a]Indicates significant difference.

$p = 0.64$), and RF ($F_{(24,264)} = 0.36$; $p = 0.99$) were not affected by TOD of testing (Table 1). The evolution of the RMS_{norm} values recorded in the knee extensor muscles demonstrated the same profile across repetitions for each muscle with a fast increase during the first 6 repetitions for RF and first 8 repetitions for VM and VL followed by a slight decrease until the 50th repetition (Figure 2).

Analysis of the antagonist activity showed that RMS_{norm} values recorded from the BF muscle demonstrated a significant increase ($F_{(24,264)} = 2.11$; $p < 0.003$) between the first and the last repetitions of $37.9 \pm 3.7\%$ and $38.3 \pm 1.8\%$ at 06 : 00 h and 18 : 00 h, respectively (Figure 2). The RMS_{norm} variations of the BF were not affected by the TOD ($F_{(24,264)} = 0.68$; $p = 0.87$) (Figure 2).

Neuromuscular Efficiency

The NME decreased significantly during exercise for VM ($F_{(24,264)} = 150.29$; $p < 0.001$), VL ($F_{(24,264)} = 80.47$; $p < 0.001$), and RF ($F_{(24,264)} = 50.99$; $p < 0.001$). The percent decrease between the first and the last repetitions ranged from $58.5 \pm 4.1\%$ to $70.7 \pm 1.7\%$, depending muscle and TOD of testing (Table 1). Analysis of the TOD effect on the NME decrease during 50 MVC showed that this decrease was significantly greater at 18 : 00 h than at 06 : 00 h for VM ($F_{(24,264)} = 1.60$; $p < 0.05$) and VL ($F_{(24,264)} = 2.21$; $p < 0.002$). No TOD effect was observed for RF muscle ($F_{(24,264)} = 0.68$; $p = 0.87$). *Post hoc* LSD analysis on the VM and VL NME values showed that significant differences occurred only during the first 20 repetitions (Figure 3).

DISCUSSION

The main finding of this study is that diurnal variation of the muscle capacity, traditionally observed in the literature during maximal

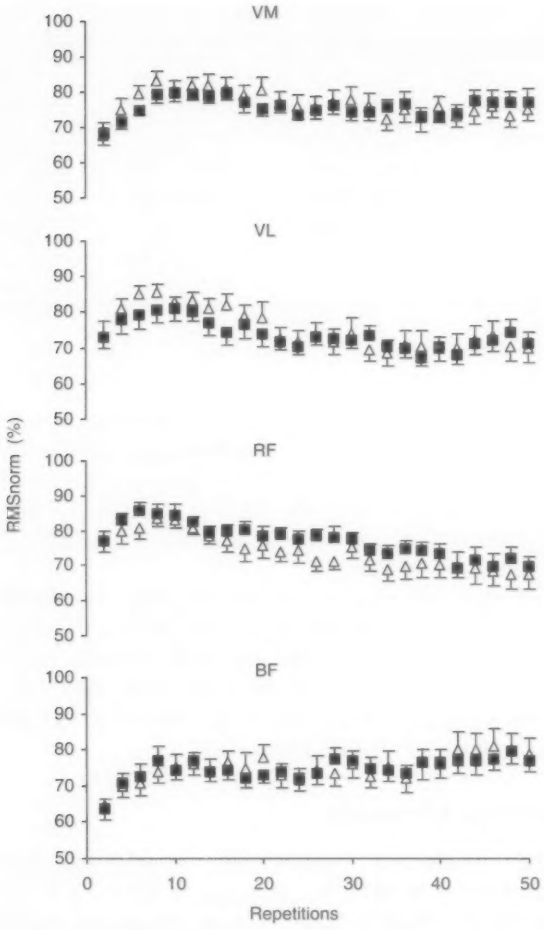

FIGURE 2 Relationship between RMS normalized values and repetition number at 06:00 (△) and 18:00 h (■) for Vastus Medialis (VM), Vastus Lateralis (VL), Rectus Femoris (RF), and Biceps Femoris (BF) muscles. Values are mean ± SE ($n = 12$).

and brief isokinetic exercises, is not maintained when exercise was prolonged.

During this study, oral temperature was recorded as a biological marker of the experimental population and was used to justify the choice of the two times of the test sessions (06:00 and 18:00 h). So, in accordance with a number of previous studies, our results demonstrated a diurnal variation in oral temperature and maximal torque values. Oral temperature increased significantly from 06:00 to 18:00 h (Reilly and

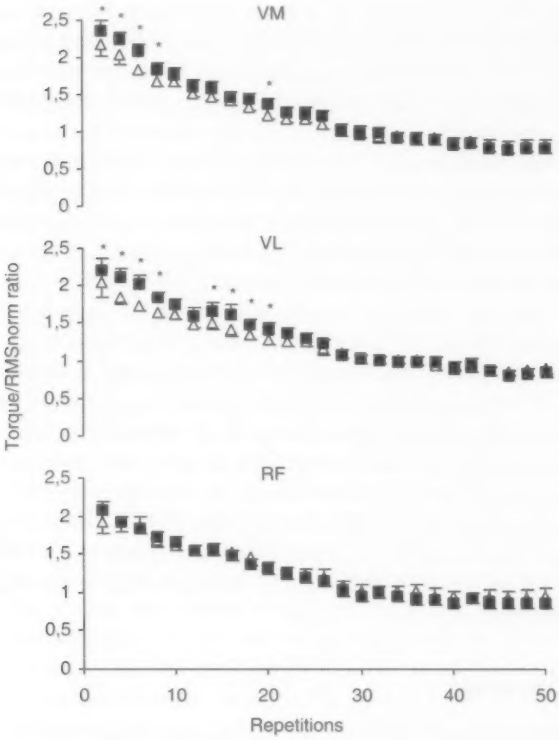

FIGURE 3 Relationship between Torque/RMS_{norm} ratio values (NME) and repetition number at 06:00 (△) and 18:00 h (■) for Vastus Medialis (VM), Vastus Lateralis (VL), and Rectus Femoris (RF) muscles. Values are mean ± SE ($n = 12$). *Indicates significant difference between 06:00 and 18:00 h.

Brooks, 1990; Melhim, 1993; Wyse et al., 1994; Gauthier et al., 1996), and the amplitude of the temperature fluctuation recorded in our study (0.88°C) is similar to the amplitude of 0.76°C and 0.8°C recorded by Ilmarinen and colleagues (1980) and Reilly and Down (1992), respectively. In the same way, the present results indicate a significant increase in T_{45}Max in the evening (18:00 h), in comparison with the morning (06:00 h). This is in accordance with Wyse and associates (1994) and Gauthier and coworkers (2001), who showed a circadian variation in muscle strength during concentric contraction with the amplitude ranging from 5.33 to 12.03%, depending on the muscle and/or angular velocity. Nevertheless, our results were not in accordance with Cabri and associates (1988), who failed to observe this circadian variation during concentric contraction.

Among the factors frequently presented in the literature to explain the diurnal variation of the muscular torque, some authors have postulated the hypothesis of a causal link between core temperature and muscular torque fluctuation (Atkinson et al., 1993; Bernard et al., 1998). Bergh and Ekblom (1979) showed that muscular torque decreased by nearly 5% when muscle temperature decreased by 1°C. Febbraio and colleagues (1996) showed that an increase of 1.9°C in muscle temperature resulted in an increase of muscle glycogenolysis, glycolysis, and high-energy phosphate degradation during exercise. They hypothesized that an increase in muscle temperature elevated the rate of ATP turnover associated with the exercise and/or changes in the anaerobic/aerobic contribution to ATP resynthesis. Thus, the lower muscular torque observed in the early morning could be partly explained by the lower core temperature at this time. However, Martin and coworkers (1999) have shown that, despite an artificial heating of the adductor pollicis muscle by 5°C at 07 : 00 h, the diurnal fluctuation in muscle strength, nonetheless, persisted. Furthermore, these authors have proven, using the interpolated twitch method, that neural command is not affected by the TOD. They concluded that diurnal variation of muscle strength is not completely mediated by core temperature variation, and that other changes affecting peripheral processes are involved.

Torque Decrease

The torque decrease observed in the present study was in accordance with those reported by Deschenes and coworkers (1998) (−49.7%) and Perry-Rana and colleagues (2002) (−65%) after such an exercise. The decrease profile of the torque observed at 06 : 00 h and 18 : 00 h is the same as those described by Gerdle and Elert (1994), Lindtrom and coworkers (1995) and Perry-Rana and colleagues (2002), with an initial important decrease during the first contractions, followed by a slight decrease thereafter. Lindstrom and coworkers (1995) used the term of "fatigue phase" to illustrate this first phase during which torque values decrease quickly, and the term of "endurance phase" to illustrate this second phase when torque values reach a floor.

Our experiment shows that torque decrease during the 50 MVC is TOD dependent, with a higher torque decrease when exercise is performed at 18 : 00 than at 06 : 00 h. These results are not in accordance with those of Deschenes and colleagues (1998), who did not observe significant TOD effect on muscle fatigue during a similar exercise composed of 50 MVC of the knee extensors and flexors. However, during this study, the experimental protocol involved performing alternating maximal concentric muscle actions of the knee flexor and extensor muscles, and furthermore, the angular velocity was higher ($3.14\,\text{rad}\cdot\text{sec}^{-1}$). In our

experiment, the slower angular velocity used ($2.09\,rad \cdot sec^{-1}$), results from a greater test duration. Furthermore, subjects had no alternating antagonist movement (knee extension and flexion) as in the study by Deschenes and colleagues (1998), which focused on knee extension movement only.

Post-hoc analysis demonstrate that significant torque value differences between 06 : 00 and 18 : 00 h occur until the 26th repetition, corresponding to the "fatigue phase" previously described by Lindstrom and associates (1995), when torque values decreased rapidly. As well, decrease in torque values slows down to reach a floor that appears to be constant and independent of the TOD of testing.

Pincivero and coworkers (2001) suggest that the ability to generate and maintain a maximal torque level for a 30 sec period—and thus during this first "fatigue phase"—is mainly driven by anaerobic sources of which a significantly large contribution is from high energy phosphates and glycolytic metabolism. Assuming this first period is dependent on anaerobic sources, the higher torque values recorded at 18 : 00 than at 06 : 00 h are in accordance with Melhim (1993) and Souissi and colleagues (2002), who observed a TOD effect in anaerobic performance with peak values reached at 18 : 00 h. These authors suggest that the increase of the core temperature during the day exerts significant effect on anaerobic processes, with better enzymatic functioning, as we described previously. Thus, our results showed that torque values were higher at 18 : 00 than at 06 : 00 h during a period of approximately 40 sec (26 repetitions of 1.5 sec duration each), and thus could partially explain diurnal variations of maximal anaerobic power and mean anaerobic power during the Wingate test previously described by these authors.

Electromyographic Parameters

In accordance with previous reports (Moritani et al., 1986; Pasquet et al., 2000; Perry-Rana et al., 2002), the torque decrease observed throughout the exercise was accompanied by changes in the collected EMG data. During the fatiguing exercise, an increase in the RMS_{norm} values was observed in agonist muscles during the first repetition. After this point, RMS_{norm} values slightly decreased until the end of the exercise session. This electromyographic pattern could be attributed to fatigue-induced changes in motor unit recruitment and/or motor unit discharge rates (Moritani et al., 1986; Pasquet et al., 2000; Perry-Rana et al., 2002).

Concerning TOD effect, results are in accordance with Martin and associates (1999) and Castaingts and colleagues (2004), who did not observe significant fluctuation in electromyographic parameters during maximal voluntary contractions. The absence of a significant diurnal variation of this parameter suggests that central motor command failure did

not play a key role in the diurnal variation observed on the torque decrease during the fatigue test.

For antagonist activity, the RMS_{norm} values of the BF muscle increase throughout the 50 MVC. The rise observed in this antagonist muscle activity gives evidence of the active BF involvement during knee extension. Previously, Kellis (1999) suggested the important role of the BF muscle in the stability of the knee joint during extension movements, but he showed that during a bout of concentric MVC of the knee extensors at an angular velocity of $2.09\,rad \cdot sec^{-1}$, the variation of the antagonist activity in the resultant joint moment was not significant. However, the exercise consisted of only 34 repetitions. In our study, increase of the RMS_{norm} values of the BF could, at least in a small way, contribute to the decrease of the resultant joint moment. Considering the absence of diurnal variation in BF RMS_{norm} values, the diurnal variation observed in fatigue could not be explained by variation of the antagonist electromyographic activity and thus by diurnal variation of the central command.

Neuromuscular Efficiency

The torque decrease observed during our experiment was not paralleled by a similar decrease of the RMS_{norm} values recorded from the three agonist muscles. As a consequence, the NME, calculated with the Torque/RMS_{norm} ratio, was significantly reduced during the exercise. Thus, the torque decrease observed during 50 maximal isokinetic contractions could be mainly attributable to peripheral fatigue (Bigland-Ritchie, 1978). The peripheral fatigue probably involved several sites, but intracellular processes associated with excitation-contraction coupling are one of the sites most involved (Duchateau and Hainaut, 1986). Metabolic and ionic changes are mainly responsible for the inhibition of the excitation-contraction processes and thus are at the origin of the developed peripheral fatigue (Westerblad et al., 1991).

The NME values of the VL and VM muscles were initially higher at 18 : 00 than at 06 : 00 h. Callard and coworkers (2000) previously obtained similar results on the quadriceps muscle with NME values higher in the evening than morning during a submaximal exercise. However, our results show for the VL and VM muscles that during 50 MVC (i) the NME decrease is higher in the evening than morning and (ii) diurnal differences in the NME values occurred during the first 20 repetitions only. These observations can be related to the two different phases previously described in the diurnal variation of the decrease in T_{45} values. In this way, NME values of the VL and VM muscles are higher at 18 : 00 than at 06 : 00 h during the first phase, and were not TOD-dependent when exercise was prolonged. The diurnal variation of the NME decrease during the initial phase shows that peripheral mechanisms involved in

muscle contraction can be differently affected by fatigue, depending on the TOD. Thus, diurnal variations observed in MVC would be ascribed to peripheral changes rather than alterations in central factors, such as the ability to activate muscle twitches (Martin et al., 1999). The possible mechanisms underlying peripheral changes are still unknown, but several hypotheses have been proposed by Martin and colleagues (1999). Some modifications in the excitation-contraction coupling could be involved through (i) enhancement of the Ca^{2+} released by the sarcoplasmic reticulum (Duchateau and Hainaut, 1986), (ii) increase in the Ca^{2+} sensitivity of the contractile proteins (Palmer and Moore, 1989; Pate and Cooke, 1989) and (iii) enhanced myosin ATPase activity (Barany, 1967). These peripheral changes, which occur during the day, are a series of phenomena that could be an explanation of the maximal voluntary force enhancement that is seen throughout the day and, likewise, an explanation of the diurnal variation of the muscle fatigue observed in this experiment.

CONCLUSION

In conclusion, muscle fatigue induced by maximal and repeated isokinetic exercise was deemed to be greater in the evening than in the morning. However, this diurnal difference appeared to be significant during the initial fatigue phase only, when torque values decrease rapidly. Next, in the final phase, muscle performance reaches a floor value that appeared to be constant with the TOD. These diurnal variations are paralleled with the diurnal variation of the neuromuscular efficiency of VM and VL muscles, suggesting that peripheral mechanisms involved in muscle contraction can be differently affected by fatigue depending on the TOD.

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Chronobiology International, 22(6): 1013–1027, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500397900

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ACUTE CHANGES IN KNEE-EXTENSORS TORQUE, FIBER PENNATION, AND TENDON CHARACTERISTICS

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The aim of the current study was to examine the relationships between quadriceps torque, vastus lateralis pennation angle (θ), and patella tendon stiffness (K) at 07:45 and 17:45 h. Using short-duration static contractions, simultaneous recordings were made of vastus lateralis (VL) electromyograph (EMG), θ and patella tendon K. Peak isometric extension torque (Peak torque Ext_{corr}) increased by $29.4 \pm 6.5\%$ at a knee angle of 70° ($p = 0.03$) in the evening compared to the morning. In the contracted muscle, a $35.0 \pm 11.0\%$ ($p = 0.02$) time-of-day (TOD)-related change in θ (to a greater evening compared to morning θ) was observed. Morning and evening measures of θ were also made, both at rest and at a standardized force level (250 N), to separate architecture change effects from increased torque capacity effects. Significant increments in θ in both the resting muscle ($13.0 \pm 5.1\%$, $p = 0.046$) and during the standardized exertions ($8.0 \pm 3.1\%$, $p = 0.04$) were observed in the evening versus the morning. Increases in θ with TOD were significantly correlated with the 40% ($p = 0.018$) decrease in K both during the standardized contractions ($r = 0.788$, $p < 0.001$) and at rest ($r = 0.77$, $p = 0.026$). These data show that TOD affects K and θ and that these two important factors involved in *in-vivo* muscle torque generation capacity are associated. The data also show that despite the potentially deleterious effects of the direction of the changes in both K and θ with TOD, peak torque Ext_{corr} still shows a significant upward shift in the evening relative to the morning.

Keywords Diurnal variation, Time-of-day, Optimal torque, Muscle architecture, Series elastic element

Submitted June 13, 2005, Returned for revision August 12, 2005, Accepted August 23, 2005
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INTRODUCTION

Previously, the time-of-day (TOD) dependence of muscle strength has been investigated in detail as seen in studies of lower limb performance (Deschenes et al., 1998; Gauthier et al., 1996; Martin et al., 1999; Wyse et al., 1994), with all these studies reporting that peak strength is increased over the time course of the day. In addition, Drust and colleagues (2005) recently reported that sporting performance, which is in part reflective of changes in muscle output characteristics, is TOD-dependent. However, no attempt to study the effect on the TOD/strength relationship in conjunction with internal muscle structure has previously been made. This is indeed surprising since the evidence for muscle internal structure changes being an important factor in the development of torque have previously been documented (Maganaris, 2001; Narici et al., 1996).

It is generally known that the level of myofilament overlap in a sarcomere controls the level of contractile force (Huxley, 1957). It has previous been demonstrated (Gordon et al., 1966) that in isolated muscle fibers, the length-tension relationship approximates a bell-shaped curve. *In vivo*, however, the picture is more complex in that one has to take into account not only anatomical levers, but also muscle architecture as defined by fiber pennation angle, as well as the in-series elastic component of the whole structure (Narici et al., 1996), the latter being composed of both aponeuroses and tendon structures. Previous authors (Maganaris, 2001, 2003) have investigated how the joint torque-angle relationship relates to muscle architecture. These studies showed that the external torques generated by the muscle decreased as the muscle fiber pennation angle increased.

Muscle physiological cross-sectional area (pCSA), which is determined by dividing muscle volume by fascicle length (F_L) and then multiplying the result by the cosine of the pennation angle (θ), has been shown to reflect the force-generating capacity of the muscle (Maganaris et al., 2001; Narici et al., 1996). Previous studies have reported a relationship between pCSA and expression of strength (Bamman et al., 2000). However, if fiber pennation angle is significantly affected by TOD, studies that have examined the chronic effect of training on pCSA to examine the relationship with force changes (Aagaard et al., 2001; Reeves et al., 2004) may be confounded if investigators do not control or account for this effect. Moreover, expectations are for a stiffer tendon to promote smaller changes in fiber pennation angle for a given fiber force in contrast to a compliant tendon. Indeed, it is reported (Kubo et al., 2001) that with an increase in tendon compliance, the muscle fiber pennation angle also increases. Furthermore it is likely that collagenous tissue undergoes remodelling after usage, and this "remodelling may involve a period of ultrastructural weakness" (Zamora and Marini, 1988).

However, no reports have thus far addressed the possibility that tendon characteristics might change over the course of the day.

The purpose of the current study was to investigate: 1) the effect of TOD on both patella tendon stiffness and vastus lateralis fiber pennation angle, and 2) the relationship between any relative changes in fiber pennation angle, tendon stiffness, and torque. It was hypothesized that tendon stiffness would decrease from morning to evening and that this effect would be concomitant with changes in muscle fiber pennation and torque output of the knee extensors.

METHODS

Subjects and Experimental Design

Study subjects were 13 young men aged 24.0 ± 4.6 yrs, height 1.72 ± 0.03 m and weight 74.3 ± 5.1 kg. Participants were selected on a "moderately morning" ($n = 6$) to "intermediate" ($n = 7$) type on the basis of their answers to the "Morning-eveningness in human circadian rhythms" questionnaire (Horne and Östberg, 1976), and were rejected if they were "moderately evening" to "evening" types, in order to control for the effects of self-motivation in the trials. Subjects had no history of lower-limb injury that might have prevented them from participating in the study. Morning experiments started at 07:45 h, while evening experiments started at 17:45 h, inclusive of preparation time. Subjects were asked not to participate in any physical exercises throughout the day. Experimental design was a counterbalanced type to prevent order effects, 6 subjects were tested in the order morning to evening, while the other subjects were tested in the order evening to morning. Some of the variables were measured in a sub-sample of the total group (see Results section). All the following measures were made both morning and evening and were preceded by a standardized warm-up (4 dynamic extensions/flexions over the angle range tested, see below, at 1.05 rads $\cdot$ s^{-1} and 3 isometric contractions at 90° knee angle, with full knee extension = 0°) on the isokinetic dynamometer. The investigation was approved by the Manchester Metropolitan University Institutional Ethics Committee, and all subjects gave their written informed consent to participate in the study. All procedures used in the current study conformed to the Declaration of Helsinki and to the standards of the Journal (Touitou et al., 2004).

Measurement of Maximal Knee Joint Torque

To determine the knee angle at which maximal isometric torque occurred, maximal isometric knee-extension and flexion torques were measured using an isokinetic dynamometer (Cybex, Phoenix Healthcare

Products, UK) on the right leg, at 5 angles, including 90°, 80°, 70°, 50°, and 30° knee angles. The hip was set at 85° (with full supine position = 0°); straps were positioned at the hip, shoulders, and over the right thigh to prevent any extraneous movement during contractions. Subjects were university students who had previously been tested on the Cybex on at least 3 previous occasions and thus were familiar with the procedures involved. Torque and angle were displayed on a computer screen (Macintosh G4, Apple Computers, Cupertino, CA, USA), which was interfaced with an A/D system (Acknowledge, Biopac Systems, Santa Barbara, CA, USA) with a sample frequency of 500 Hz. A total of 3 extension and flexion efforts were made at each test angle, with extension alternated with flexion contractions. Each contraction was held for 2 to 4 sec at the plateau, with a period of 30 sec given between the flexion and extension contractions, and an interval of 90 to 120 sec between each pair of contractions. The order of knee angle was randomized so as to minimize any position-related fatigue bias. Peak torque was averaged over a 1 sec period. The data from the knee angle at which the highest torque (peak isometric extension torque, PIET) was generated were used for further analysis.

Electromyographical Activity

Skin at the site of electrode attachment was shaved, abraded, and cleaned with an alcohol-based solution in order to minimize resistance. A pair of self-adhesive Ag-AgCl electrodes ~15 mm in diameter (Medicotest, Rugmarken, Denmark) were placed in a bipolar configuration, with a constant between-electrode distance of ~22 mm, at the distal one-third of the length of the muscle. These electrodes were placed in the mid-sagittal plane of the biceps femoris, while the reference electrodes were placed on the lateral tibial condyle, following a previously described method (Reeves et al., 2003a). The location of the electrodes was kept constant in the morning and evening conditions by marking the skin with a pen, with the understanding that the subject was not to clean the skin before the evening test also when the test was repeated on subsequent days, by tracing these placements on transparent acetate sheets, ensuring that an anatomical landmark and the line of the femur were recorded on the same sheet. The raw electromyographical (EMG) signal was sampled at 2000 Hz, pre-amplified (×2000), and band pass filtered between 500 Hz and 10 Hz (Biopac Systems).

Co-contraction Torque

To obtain a more accurate estimate of the knee extensor torque and to determine force in the patellar tendon, the hamstrings co-contraction torque was estimated and added to the observed knee extensor torque.

The root mean square (RMS) EMG activity corresponding to the peak torque period was analyzed over 12.5 ms epochs and averaged for a 1 sec peak torque duration. The reported EMG activity in this study corresponds to the RMS after removal of baseline values. The EMG of the long head of the biceps femoris muscle was measured to ascertain the level of antagonistic muscle co-contraction during the required isometric knee extension performances. The assumption was made that the biceps femoris was representative of its constituent muscle group (Carolan and Cafarelli, 1992). A linear relationship between knee flexor torque and biceps femoris EMG has been reported previously (Lippold, 1952; Reeves et al., 2004). Based on this relationship, the flexor torque during a knee flexion contraction was calculated, similar to the method described by Reeves and colleagues (2003b) to allow the percentage co-contraction to be determined. Briefly, the biceps femoris EMG activity during knee extension is divided by the biceps femoris peak flexor EMG; the maximal flexor force is then multiplied by this value to determine co-contraction torque. The co-contraction torque values are then used to correct the voluntary knee extension torques at each specific angle using the following formula:

$$\text{Peak torque } \text{Ext}_{\text{corr}} = \text{PIET} + \text{T}_{\text{flex}}$$

where Peak torque Ext_{corr} = corrected extensor torque, PIET = observed knee extensor torque (*i.e.*, the recorded torque during testing), T_{flex} = calculated flexor torque during knee extension (*i.e.*, co-contraction torque).

Measurement of Muscle Architecture, Tendon Cross-Sectional Area and Stiffness

The vastus lateralis fascicle pennation angle (θ) was determined using real-time ultrasonography (AU5, Esaote Biomedica, Italy) at rest, during maximal knee extensor contractions and at a standardized level of force (250 N) at the optimal knee angle. Pennation angle was measured as the angle of fascicle insertion into the deep aponeurosis, *i.e.*, the fascia separating vastus lateralis and vastus intermedius muscles (Rutherford and Jones, 1992). Images were obtained perpendicular to the dermal surface of the vastus lateralis muscle and oriented along the longitudinal plane of the muscle. Images were taken at mid-belly of the right limb using a 7.5 MHz linear array probe, 38 mm wide. Mid-belly was defined as the point along the median longitudinal axis of the muscle halfway between the distal and proximal apexes of the myotendinous junction. Images were captured at 31 frames/sec using a digital video recorder (SONY, Japan). Angle measurements were made on the digitized images using

Image J (Wayne Rasband, National Institute of Health, USA). In order to blind the investigator to the time-of-day and the angle, digitized frames were coded. The code was only revealed to the investigator after all the analyses were completed.

Tendon stiffness was determined using a previously described method (Maganaris and Paul, 1999). Briefly, in order to assess patella tendon stiffness, ramped (to maximal contraction) isometric knee extension contractions were carried out at a knee angle of 90° over a 4 to 6 sec time period. Force and ultrasound data were synchronized in order to align the records. All measurements of the tendon were carried out after 4 preconditioning trials to ensure reproducibility (Kubo et al., 2002; Reeves et al., 2003b). The ultrasound probe was positioned in the sagittal plane over the patella tendon. To detect any extraneous movement of the probe with respect to the tendon, an external marker was attached to the skin (see Figure 3); any movement of this marker would indicate relative movement of the probe. The scan was repeated if any such movement was noted. The tendon excursion was determined at intervals of ~10% of the maximal force. The average of two trials was taken to measure tendon elongation for the subject at the specific session.

Tendon forces were determined by dividing the corrected torque by an estimate of the patella tendon moment arm length at 90° (Krevolin et al., 2004). The loading phase of the force-elongation relationship was fitted with a second order polynomial function ($Y = a \cdot x^2 + b \cdot x$, where a and b are constants). Tendon stiffness (K in $N \cdot mm^{-1}$) was calculated from the gradient of the tangent at a force level (~5400 N, see Figure 4) that corresponded to the position at which the change in elongation was met with a visibly greater change in force (compared with previous rates of change). Both tendon length and cross-sectional area (CSA) were assessed in the resting state at a knee joint angle of 90° after the warm up contractions. Tendon length was measured from the apex of the patella to the superior aspect of the tibial tuberosity. Axial-plane ultrasound images taken at 50% of tendon length were utilized to determine tendon CSA. Three measurements were averaged and the mean was used for calculation of Young's modulus, which was calculated by multiplying the stiffness value by the ratio of tendon length to tendon CSA.

Reliability of Measurements

The optimal knee angle (70°) was chosen to calculate reliability of measurements. Tests were performed on separate days by the same examiner (inter-day reliability) on a sample ($n = 5$) of the study population. Intra-class correlation coefficients (ICCs) were calculated using a one-way random effects model to quantify the reproducibility of the measures (Table 1). The statistical power of all performed tests was ≥ 0.8.

TABLE 1 Repeatability of the Measures at the Optimal Knee Angle

Measure	ICC
Peak torque Ext_{corr} (Nm)	0.98
Biceps femoris RMS EMG (mV)	0.99
Pennation angle (°)	0.97
Tendon elongation (mm)	0.98

All values are ICCs (see methods). RMS, root mean square; EMG, electromyographical activity. NB. Except for tendon elongation error which was measured at 90° knee angle, the rest of the reliability tests refer to the knee at 70°.

Statistical Analysis

Data were analyzed using repeated measures two-way ANOVA. *Post hoc* tests were carried out using Student *t*-tests for paired-samples; Bonferroni corrections were applied where required. The Pearson product correlation was used to compare the degree of association between variables. Significance was set to $p < 0.05$. All data are presented as mean ± standard error of the mean.

RESULTS

The optimal angle for maximal torque generation was found to be 70° in all subjects tested. The PIET data (whether uncorrected or corrected) of the subjects showed no significant shift in optimal angle with TOD ($p > 0.05$). Figure 1A ($n = 6$) shows that the uncorrected extensor torque (PIET) at 70° incremented by 10.8 ± 4.2% ($p = 0.08$) in the evening compared with the morning. The level of antagonistic torque exhibited a tendency for a TOD effect with mean increase from 9.9 ± 1.9% to 27.6 ± 7.5% (morning to evening, $p = 0.06$). When antagonistic torques were taken into account to estimate peak torque Ext_{corr} (Figure 1B, $n = 6$), the upward shift in peak torque increased to 29.4 ± 6.5% ($p = 0.03$). In the evening session, the group torque values changed from 291.4 ± 9.2 Nm to 401.2 ± 26.8 Nm post-corrections.

In the evening, muscle fibers at optimal knee joint angle were significantly more pennate, both at rest and during peak contracted conditions. A typical example of change in resting pennation angle morning *vs.* evening is shown in Figure 2. In the evening compared to morning condition, the relaxed muscle pennation angle was augmented by 13.0 ± 5.1% ($p = 0.046$), and in the peak contracted condition the relationship was significantly increased (since increments in force were also observed in the evening compared to morning) by 35.0 ± 11.0%

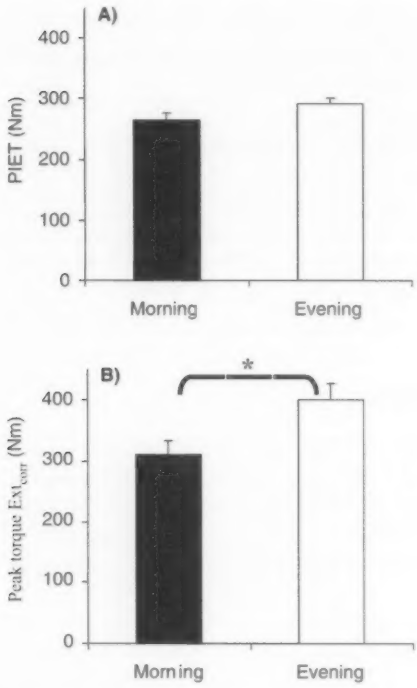

FIGURE 1 Knee extensors torque in morning (solid bars) evening (open bars). (A) Uncorrected knee extensor torque (PIET). (B) Peak torque Ext_{corr}. Values are mean ± S.E.M. ($n = 6$).

($p = 0.02$, $n = 6$). Interestingly, in the standardized exertions (250 N), a significant increment of 8.0 ± 3.1% ($p = 0.04$, $n = 11$) in pennation angle was also observed in the evening compared to the morning. It is notable that the level of antagonist co-contraction tests. This level of standardized force was not significantly different ($p = 0.898$) in the morning (3.9 ± 0.71%) compared to evening (3.8 ± 1.09%) tests.

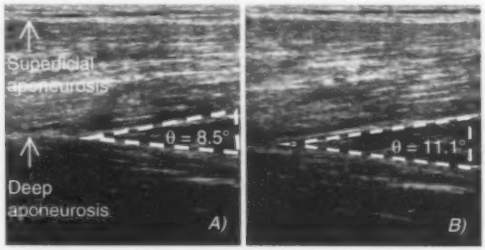

FIGURE 2 Typical sonographs of the sagittal plane of the vastus lateralis muscle at rest, with knee at an angle of 90°. (A) morning and (B) evening showing pennation angle (θ).

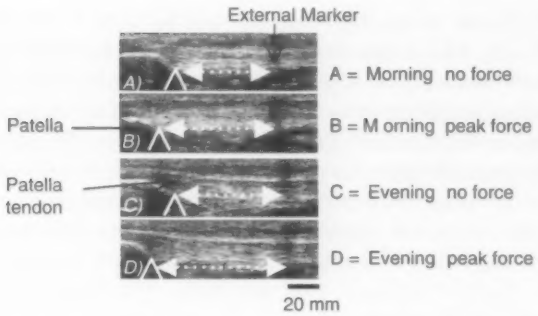

FIGURE 3 Example showing patella tendon excursion between relaxed and maximal isometric contraction at 90° knee angle in one subject over the course of a day. Short white arrows indicate displacement of apex of patella during contraction with respect to the shadow cast by the external marker fixed on the skin. The white double ended arrows show the distance between apex and external marker: A = 2.19 cm, B = 2.31 cm, *i.e.*, Morning elongation = 0.12 cm, C = 2.22 cm, D = 2.7 cm, *i.e.*, Evening elongation = 0.48 cm.

Figure 3 illustrates a typical ultrasound record of tendon elongation measurement in both morning and evening conditions. It is obvious from these images that the amount of displacement is larger in the evening than morning. The average tendon length in our group was 65 ± 4 mm, while the average tendon CSA was $86 \pm 4\,mm^2$. These values did not change significantly in the evening relative to the morning test. In Figure 4, group stiffness values significantly decreased by 40% ($p = 0.018$) from $1658 \pm 191\,N \cdot mm^{-1}$ (Young's modulus

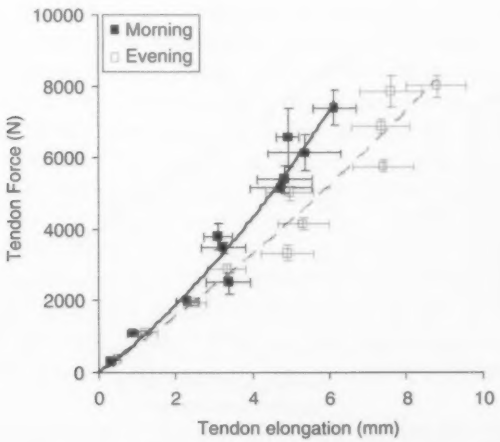

FIGURE 4 Change in patella tendon stiffness with time of the day. (*open squares*) Evening stiffness (r = 0.97) and (closed squares) morning stiffness ($r = 0.98$). Data are mean ± S.E.M. ($n = 13$).

1.2 ± 0.1 GPa) in the morning to 994 ± 189 $N \cdot mm^{-1}$ (Young's modulus 0.8 ± 0.1 GPa, *i.e.*, 35% reduction, $p = 0.040$) in the evening.

The above results show that the increased tendon compliance in the evening, relative to the morning, was accompanied by increases in pennation angle. Figure 5 illustrates the significant relationship between changes in tendon stiffness in relation to pennation angle for the standardized-load contractions ($r = 0.88$, $p < 0.001$). In addition, changes in resting pennation angles also showed significant linear correlation with associated changes in tendon stiffness (results not shown, $r = 0.77$, $p = 0.026$).

DISCUSSION

The current investigation aimed at studying the *in vivo* effect of TOD on: 1) quadriceps muscle isometric torque (peak torque Ext_{corr}) at the optimal knee joint (with 0° being a straight knee), 2) vastus lateralis fiber pennation angle, and 3) patella tendon stiffness. The optimal knee joint was consistently found to be 70° in all subjects. Our results, in terms of the average magnitude of the torque change at this knee angle between the 07:45 and 17:45 h sessions (29.4 ± 6.5% torque increment), are greater than those reported in previous studies of increment in torque with time of day. For example, an 8.9 and 7.7% increment in the maximum voluntary contraction of the adductor pollicis and knee extensor muscles, respectively, was reported (Martin et al., 1999; Nicolas et al., 2005).

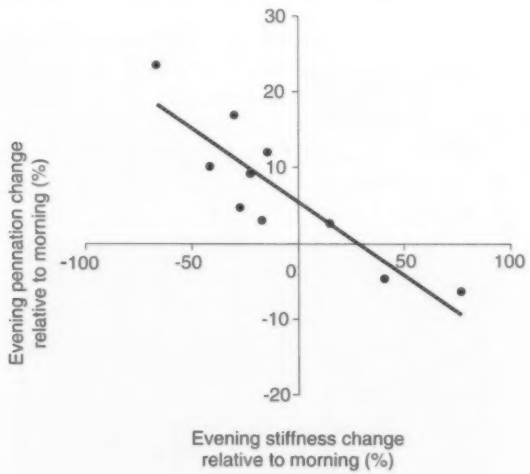

FIGURE 5 Relationship between the relative changes in patella tendon stiffness and vastus lateral is (VL) muscle pennation angle. Changes represent relative evening values as a percentage of morning values ($n = 10$). The linear relationship is described by $Y = -0.19x + 0.05$ with $r = 0.88$ ($p < 0.001$).

This difference could be explained at least in part by the method used to calculate level of torque; here co-contraction was taken into account. We have shown that in this group of subjects, co-contraction showed a strong trend towards an increase over the time course of the day. It should also be stressed here that because the young subjects used in this study carried out similar levels of habitual physical activity, and because they were asked to not perform any physical exercise between the measurement sessions the possibility that the changes in torque observed here were due to "between testing sessions" activity levels is unlikely. Nevertheless, future studies would benefit from repeating this experiment with a "bed-rest" condition in order to separate hormonal and activity level effects.

We propose that, with the ability of the knee extensors to generate greater torque in the evening, the neuromuscular system attempts to counteract the potential increase in shear torque around the knee by increasing co-contraction. Others have also noted high co-activation levels during isometric contractions at similar angles (Kubo et al., 2004), which would further support the notion that co-contraction may be an adaptive response to increased levels of joint torque.

This study is also the first to show not only a significant TOD-related increment in tendon compliance (or decrease in stiffness), but also concomitant increases in contracted fascicle pennation angles. The morning values of tendon stiffness in the current study are in line with previous reports (Reeves et al., 2003a; Reeves et al., 2003b). We discount the possibility that the tendon stiffness decrease observed with TOD was caused by any temperature influence, since previous work showed that "icing and hot pack application did not change the mechanical properties of muscle and tendon" (Kubo et al., 2005). Instead, we propose that one mechanism for the observed increase in compliance with TOD may be related to the contractile history of the muscle. Indeed, studies show that fatigue (Vigreux et al., 1980) and even simply repeated muscle contractions (Kubo et al., 2001) make tendon structures more compliant. With increased TOD, our subjects were bound to have used their muscles more than in the morning test, hence possibly causing the tendon to become more compliant. Another factor for the mechanism behind the increment in tendon compliance with TOD may be the pulsatile production of testosterone, which peaks in the morning and declines in the early evening (Deschenes et al., 1998). Animal studies have shown that increased levels of anabolic steroids produce a stiffer tendon (Miles et al., 1992), which is in general agreement with our observation of higher tendon stiffness in the morning compared with the evening tests.

The vastus lateralis was used as it was considered, in the light of previous studies (Carolan and Cafarelli, 1992; Narici et al., 1992; Reeves et al., 2004), to be a valid representative of the rest of the quadriceps muscles. The mean

fiber pennation angles reported here are in general agreement with previous reports on the *in vivo* architecture of the vastus lateralis, which noted for instance that at a knee angle of 70° the average resting pennation angle was ~15° (Fukunaga et al., 1997); this is similar to the current study values of pennation angles of 13 ± 5° for that knee angle. However, where pennation angle is measured over a period of time, as in training studies, *e.g.*, Rutherford and Jones, 1992, if time of day is not accounted for, then there is a potential for an approximately 13% error in the measurement. This might explain why Rutherford and Jones (1992) observed no relationship between pennation and training-induced increments in torque-generating capacity, a conclusion which is very much in conflict with previous reports (Aagaard et al., 2001; Narici et al., 2003).

Previous authors (Aagaard et al., 2001) reporting on the training of the vastus lateralis, showed relative changes of ~34% in pennation angle. Similarly, other training studies (*e.g.*, Kawakami et al., 1995) report pennation increments of ~29% in the biceps brachii. Our results show that it is possible that the effect in any of these previous studies might have been over/under-estimated by factors up to 13.0 ± 5.1% in terms of resting pennation angle, depending on whether on not measurement times (of day) were controlled. These corrections are not negligible and would have significant impact on the conclusion about training-induced changes on skeletal muscle characteristics.

We propose that the observed increments in pennation angle in the evening are consistent with the changes in the stiffness/compliance characteristics of the in-series elastic element. As reported in previous investigations (Kawakami and Lieber, 2000; Loren and Lieber, 1995) and those using computer models (Trestik and Lieber, 1993), all things being equal (*i.e.*, sarcomere length-tension, force-velocity properties, and optimal sarcomeres length-tension relationship being similar), a more compliant tendon would be expected to allow greater sarcomere shortening in contrast to a stiffer tendon when submitted to similar muscle forces. It is reported that in the elbow flexors, the compliance of the series elastic component is inversely related to force production (Valour and Pousson, 2003). However, in our current study we observed that although compliance significantly increased with TOD, the ability to generate torque increased significantly. At the single fiber level, all things being equal, if pennation angle increases the effective pull of the fiber on the tendon will decrease as a factor of the cosine of the pennation angle ($F_t = F_f \cdot \cos\theta$, where F_t is the force along the tendon and F_f is the fiber force). However, at the whole muscle level the pCSA of the muscle would increase if either fiber length reduces and/or pennation angle decreases ($pCSA = V/F_L \cdot \cos\theta$). Since pCSA has been shown to positively correlate with strength (Bamman et al., 2000) so that $r = 0.715$ ($p < 0.05$), an increment in pCSA could potentially affect the relationship between pCSA and force

generation capacity, particularly if the pCSA increments do not exhibit a proportional increment in force.

In addition, we have shown that both the contractions standardized at 250 N (to separate the effects of increase in force from an alteration in the mechanical behavior of the muscle-tendon complex), and also at rest, pennation angle is increased in the evening relative to the morning, an increment which would result in shorter sarcomeres. We should emphasize here that the standardized loads in the morning and the evening sessions were equivalent since the difference in antagonist co-contraction between morning and evening at this level of force was not significant (0.13% lower in the evening, $p = 0.898$), hence validating the assumption that the agonist muscle in the two different sessions experienced the load to the same degree. It remains, however, that the increment in pennation angle at rest could be brought about by increased resting muscle tone. Because no significant changes in vastus lateralis muscle resting electromyographical activity (results not shown) were seen in the evening compared with in the morning, we suggest that the muscle presents greater pennation angles (and possibly shorter fascicle lengths) from morning to evening mainly because of increases in-series compliance. Indeed, it can be seen from Figure 5 that changes in tendon compliance with TOD explain ~77% of the variance in pennation angle under conditions of standardized load.

In conclusion, we have shown in this study that TOD affected both tendon compliance and fiber pennation angle and that these changes were seen to be associated. We also observed that despite the potentially deleterious effects of both tendon characteristics and muscle fiber pennation changes with TOD, accurate measurements of the human knee extensor torque-angle relationship over the range of 30° to 90° knee angle show a significant upward shift between morning and evening conditions.

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Chronobiology International, 22(6): 1029–1039, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500397918

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MORNING VERSUS EVENING POWER OUTPUT AND REPEATED-SPRINT ABILITY

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We investigated the effect of time-of-day on both maximal sprint power and repeated-sprint ability (RSA). Nine volunteers (22 ± 4 yrs) performed a RSA test both in the morning (07:00 to 09:00 h) and evening (17:00 to 19:00 h) on different days in a random order. The RSA cycle test consisted of five, 6 sec maximal sprints interspersed by 24 sec of passive recovery. Both blood lactate concentration and heart rate were higher in the evening than morning RSA (lactate values post exercise: 13 ± 3 versus 11 ± 3 mmol/L^{-1}, $p < 0.05$). The peak power developed during the first sprint was higher in the evening than morning (958 ± 112 *vs.* 915 ± 133 W, $p < 0.05$), but this difference was not apparent in subsequent sprints, leading to a higher power decrement across the 5×6 sec test in the evening (11 ± 2 *vs.* $7 \pm 3\%$, $p < 0.05$). Both the total work during the RSA cycle test and the power developed during bouts 2 to 5 failed to be influenced by time-of-day. This suggests that the beneficial effect of time-of-day may be limited to a single expression of muscular power and fails to advantage performance during repeated sprints.

Keywords Circadian rhythm, Anaerobic exercise, Physical performance

INTRODUCTION

The human body is dependent on biological rhythms that influence both mental and physical activities (reviewed in Atkinson and Reilly, 1996). The effects of time-of-day on short-term anaerobic performance

Submitted 22 August 2005, Returned for revision 12 September 2005, Accepted 26 September 2005

This work was supported in part by the General Council of the Department of Guadeloupe, French West Indies.

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(<6 sec) are well documented. In most studies, a significant increase in maximal sprint performance has been observed either when tests are conducted over the entire 24 h (*e.g.*, Souissi et al., 2004), or when they are conducted during the daytime (*e.g.*, Racinais et al., 2004). In contrast, studies of the effects of time-of-day on aerobic metabolism have generally failed to report diurnal variation in oxygen uptake, especially with intensity near maximal (Dalton et al., 1997; Deschenes et al., 1998). Indeed, peak oxygen consumption (peak. VO_2) (Jones et al., 1995) and time to exhaustion (Hill et al., 1988) have both been reported not to display significant diurnal variation. Moreover, the ventilatory threshold determined during a progressive and maximal exercise test usually occurs at the same intensity in the morning and evening (Hill et al., 1989). Carter and colleagues (2002) also found no effect of time-of-day on oxygen uptake kinetics at the onset of moderate or high-intensity exercise. Thus, it appears that: (i) performance during a single, maximal sprint is higher in the evening than in the morning, but (ii) aerobic metabolism in maximal or near-maximal conditions displays the same characteristics in both the morning and evening.

To date, very few studies have investigated the effect of time-of-day on a task that is dependent on both of these qualities, such as the ability to repeatedly perform short (<10 sec), all-out sprints, separated by brief recovery periods (*i.e.*, repeated-sprint ability, RSA). To the best of our knowledge, there appears to be only one study showing that the recovery pattern of maximal power output is quite identical at 09:00 and 18:00 h, but this previous study investigated only two consecutives sprints with recovery periods lasting 30 to 120 sec (Falgairette et al., 2003). While many team sports require participants to repeatedly produce maximal or near-maximal sprints of short duration (1 to 7 sec) interspersed with brief recovery periods (Bishop et al., 2001), it is unknown whether the higher maximal sprint performance previously observed in the evening than in the morning can be maintained during several subsequent sprints. The purpose of this study, therefore, was to investigate the effect of time-of-day on both maximal sprint power and repeated sprint ability. As aerobic metabolism displays the same characteristics in both the morning and evening, and because Dawson and colleagues (1993) and Bishop and colleagues (2004) have both reported a significant correlation between peak oxygen consumption (peak. VO_2) and RSA (5 × 6 sec sprints with 24 sec of recovery), we hypothesize that the RSA should be the same at these two times of the day.

MATERIALS AND METHODS

Subjects

Nine male, diurnally active physical education students gave written informed consent to participate in this study after receiving a thorough

explanation of the protocol. The study was approved by the local ethics committee and meets the ethical standards of the Journal for the conduct of human biological rhythm research (Touitou et al., 2004). The mean (±SD) age, height, body mass, and body fat of the subjects were 22 ± 4 yrs, 1.78 ± 0.07 m, 73.5 ± 10.5 kg, and 12 ± 3%, respectively. The body fat was calculated from skinfold thickness measurements using Durnin and Womersley's Equations (1974). The subjects were all physically active (physical activity: 10 ± 8 h wk^{-1}). The subjects were classified as either "moderately morning type" ($n = 2$), "neither type" ($n = 6$) or "moderately evening type" ($n = 1$) from their responses to the self-assessment morningness/eveningness questionnaire of Horne and Östberg (1976).

Materials

Tests were conducted on a friction-loaded cycle ergometer (Monark 824E, Stockholm, Sweden). The flywheel of this ergometer has a moment of inertia of 0.42 kg m^{-2}. The braking resistive force applied on the flywheel was set at 60 g kg^{-1} of body mass on the basis of previous results showing that a load between 50 and 75 g kg^{-1} of body mass is suitable for the determination of maximal power in young adults (Doré et al., 2000). The cycle was equipped with toe-clips to prevent the subjects' feet from slipping. A disk and a photoelectric cell were fixed on the flywheel and the frame, respectively, in order to obtain the speed 12 times per flywheel revolution. The force was derived by the sum of the braking resistive force and the inertial force [calculated from the acceleration following the method of Martin and coworkers, (1997)] and the power output was calculated by multiplication of the velocity by the total force. The data were collected by an acquisition card (DAQ-Pad 6020E, National Instrument, Texas, USA) and analyzed by software developed in our laboratory with a LabVIEW interface (LabVIEW, National Instrument, Texas, USA).

Experimental Procedure

All subjects completed a strictly identical test session both in the morning (between 07:00 and 09:00 h) and evening (between 17:00 and 19:00 h). The morning and evening experiments took place on different days, separated by 3 d at most, in a counterbalanced order. The laboratory conditions were recorded by an electronic thermometer-hygrometer (Novo 16755, Novo, France, precision 0.1°C) and were 20.9 ± 0.7°C and 69 ± 4% for temperature and humidity, respectively. The subjects were asked to avoid all vigorous activity for 24 h before each test, to sleep normally, and to wear the same sportswear and shoes for all tests.

Sessions began with 1 h in resting conditions in a seated position. Several psychological (subjective mood, energy, arousal, and motivation) and physiological (body mass, blood lactate concentration [La^-], and rectal temperature [T_{rect}] parameters were measured at the end of the resting period. Subjects then performed a pre-test warm-up consisting of 5 min cycling at 84 W, followed by 3 min of rest, and followed by a 10 sec maximal sprint test. The first 6 sec of the 10 sec sprint was used as the criterion score during the subsequent RSA cycle test. Upon completion of the 10 sec test, subjects rested for 5 min before performing the RSA cycle test. During the first sprint of the RSA cycle test, subjects were required to achieve at least 95% of their criterion score, as a check on pacing (which they always did). These procedures of both warm-up and criterion score correspond to the procedure previously used to validate the RSA test of this study (Bishop et al., 2001). During the RSA cycle test, ratings of perceived exertion (RPE) and heart rate (HR) were recorded at the end of each sprint. A blood sample obtained after the final sprint was later analyzed for lactate concentration.

Psychological Indicators

During the resting period preceding the RSA test, each subject was asked to indicate their subjective mood, energy, arousal, and motivation using a cross on 4 simple visual non-graduated scales (Akerstedt et al., 1976). The scale ranged from depressed to very happy for the mood scale, from tired to full of energy for the energy scale, from asleep to completely awake for the arousal scale, and from absolutely not motivated to extremely motivated for the motivation scale. The positions of the cross were measured in order to represent scores of self-assessed mood state (the maximum possible was 14). During the RSA cycle test, the RPE was measured after each 6 sec sprint using the Borg scale graduated from 6 to 20 (Borg 1970).

Physiological Measures

After 1 h in resting conditions and in a seated position, body mass and T_{rect} were measured using an electronic scale (Teraillon, France, precision 0.1 kg) and a clinical electronic thermometer (MT 1691 BMWC, Microlife Ltd., Taiwan, precision 0.1°C, insertion depth 2 cm), respectively. The HR was measured after each 6 sec sprint of the RSA test using a heart rate monitor (Polar S610i, Polar Electro Oy, Kempele, Finland) strapped to the chest wall directly above the heart. Fingertip arterialized blood micro samples were taken with a lancet (B-D micro-Fine +, Becton Dickinson) for lactate analysis immediately at the end of the 5th sprint of the RSA test. The [La^-] was determined with an instrument for the resolution of

lactate (Accusport, Boeringer Mannheim) and a testing strip (BM-Lactate, Roche).

RSA Test

The RSA cycle test consisted of five, 6 sec, maximal sprints every 30 sec (Bishop et al., 2001, 2004). During the 24 sec recovery between sprints, subjects remained seated on the bicycle and were allowed to follow the countdown. Five sec before starting each sprint, subjects were asked to assume the ready position and await the start signal announced by a countdown given by the experimenter. Strong verbal encouragement was provided to each subject during all sprints. This test has previously been reported to be both a valid (Bishop et al., 2001) and reliable (FitzSimons et al., 1993) assessment of RSA.

Mechanical Indices

For each 6 sec sprint, peak power output (P_{peak}), power output during the last pedal revolution (P_{end}), and decrease of muscular power from P_{peak} to P_{end} were calculated. The percentage decrement over the repeated sprints for P_{peak} (P_{dec}) was also calculated (Bishop et al., 2001, 2004; FitzSimons et al., 1993). The total work (W_{total}) corresponded to the sum of the work produced during each sprint of the RSA test calculated by the integral of the power curve.

Statistical Analysis

Each variable was tested for normality using the Skewness and Kurtosis tests with acceptable Z values not exceeding ± 1.5. With the assumption of normality confirmed, parametric tests were performed. The effect of time-of-day on the RSA was verified by a two-way analysis of variance (ANOVA) with repeated measures (ANOVA, 5 sprint repetitions $\times$ 2 times-of-day). The P_{dec} was analyzed with one-way ANOVA with repeated measures (morning *vs.* evening). Pairwise contrasts were used when necessary to determine where significant differences occurred. Data are displayed as mean $\pm$SD, and statistical significance was set at $p < 0.05$.

RESULTS

Psychological Indicators

The scores of self-assessed mood state were significantly higher in the evening than morning (Table 1). Data showed that subjects had a higher subjective arousal ($F_{1,8} = 13.93$, $p < 0.05$), energy ($F_{1,8} = 5.83$, $p < 0.05$),

TABLE 1 Physiological Values and Mood States at Rest

Variable	Values (mean ± SD)		Difference	
	Morning	Evening	Percent	Significance
Mass, kg	73.6 ± 11.1	73.4 ± 10.6	0%	NS
Rectal temperature, °C	37.0 ± 0.3	< 37.3 ± 0.3	+1%	*
Mood score	9.1 ± 2	< 10.5 ± 2	+15%	*
Energy score	6.4 ± 2	< 8.7 ± 3	+36%	*
Arousal score	6.9 ± 3	< 10.9 ± 3	+58%	*
Motivation score	8.4 ± 3	< 10.0 ± 3	+18%	*

The percentage difference represents the evolution from morning to evening values in percentage of the morning values. NS = not significant, $^*p < 0.05$.

and motivation ($F_{1,8} = 12.25, p < 0.05$) in the evening and were in a better mood ($F_{1,8} = 9.32, p < 0.05$). The RPE (Figure 1) scores showed a maximum of about 17 during the RSA test, both in the morning and evening, which corresponds to very hard exercise on the Borg scale. There was a significant increase in RPE between each sprint ($F_{4,32} = 37.46, p < 0.05$), but there was no significant difference in RPE between the morning and evening, nor any significant interaction.

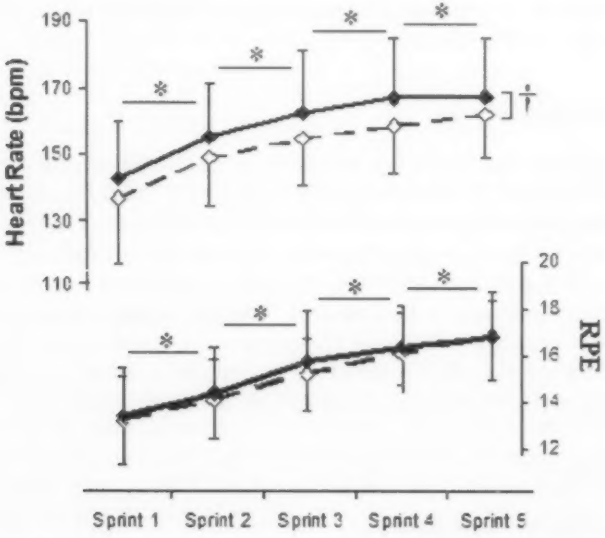

FIGURE 1 Heart rate during the 5 × 6 sec RSA test. Both RPE and heart rate increased across the 5 × 6 sec cycle test (*: significant difference between consecutive sprints, $p < 0.05$). Heart rate was significantly higher in the evening than in the morning (†: $p < 0.05$). —◆— Evening values, – –◇– – Morning values. Values are mean ± SD.

Physiological Measures

There was no statistical difference in mass ($F_{1,8} = 0.72$) between the morning and evening trials (Table 1). Resting rectal temperature (Table 1) was significantly higher in the evening than morning ($F_{1,8} = 10.20$, $p < 0.05$). Heart rate significantly increased during the RSA-test ($F_{4,32} = 65.36$, $p < 0.05$), independently of time-of-day. Heart rate (Figure 1) was higher in the evening than morning ($F_{1,8} = 5.37$, $p < 0.05$). Blood lactate concentration following exercise was also significantly higher in the evening than morning (13 ± 3 *vs.* 11 ± 3 mmol/L^{-1}) ($F_{1,8} = 5.91$, $p < 0.05$).

Repeated-Sprint Ability

As body mass was constant throughout the day (variation of 0.3%), the same results were observed for both absolute and relative values; only the absolute values are presented here. The P_{peak} values (Figure 2) decreased significantly across the 5 × 6 sec test ($F_{4,32} = 45.89$, $p < 0.05$) and there was a significant effect of time-of-day on the decrease of P_{peak} ($F_{4,32} = 3.32$, $p < 0.05$). *Post-hoc* analysis showed a higher P_{peak} during the first sprint in the evening than in the morning ($F_{1,8} = 10.98$, $p < 0.05$), and thereafter, the evening values dropped to the level of the morning values for the other sprints ($F_{1,8} < 0.311$). Therefore, the results showed that P_{dec} was higher in the evening than the morning trial ($F_{1,8} = 10.57$, $p < 0.05$). The decrease from P_{peak} to P_{end} for each

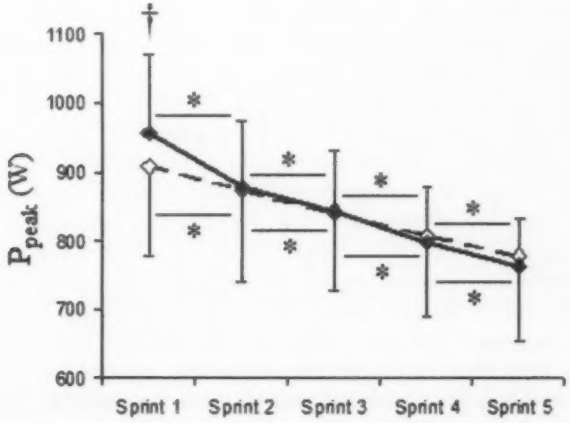

FIGURE 2 Power during the 5 × 6 sec RSA test. Peak power (P_{peak}) values decreased across the RSA test. The peak power during the first sprint was higher in the evening. —◆— Evening values, – –◇– – Morning values. Values are mean ± SD. †: Significant difference between morning and evening in the sprint 1, *: significant difference between consecutive sprints, $p < 0.05$.

sprint increased across the sprint repetition ($F_{4,32} = 6.97, p < 0.05$), independently of time-of-day. The W_{total} produced in the morning and in the evening RSA was not statistically different ($F_{1,8} = 0.01, p = 0.93$).

DISCUSSION

The major finding of the present study is that P_{dec} during a 5 × 6 sec RSA test is affected by time-of-day. It appears that muscle power is improved in the evening during the first sprint, but that there is a common end-point (fatigue) between the morning and evening when sprints are repeated. It has recently been shown that the diurnal increase in muscle power is blunted either by aging (Deschenes et al., 2002) or by a passive warm-up (Racinais et al., 2004); our results add that power output failed to be maintained higher in the evening during subsequent sprints.

Diurnal Increase in Muscular Power

The complete description of circadian rhythms requires measurements during an entire 24 h cycle, but we investigated diurnal variations in performance only during the day–time as exercise training or sports event are rarely performed during the night-time. Furthermore, the test times were chosen with regard to the literature. Bernard and colleagues (1998) showed that cycle ergometer sprint power (<10 sec) was significantly higher at 18:00 h than 09:00 h, and Racinais and coworkers (2004) observed greater cycling sprint power (<10 sec) in the evening (17:00 to 19:00 h) than in the morning (07:00 to 09:00 h). Consistent with this previous research, an increase of P_{peak} during the first sprint was observed from the morning to the evening (+5.3%) in the present study.

It has previously been suggested that the diurnal increase in central temperature may be responsible for the diurnal increase in muscular power during sprint cycling (Bernard et al., 1998; Coldwells et al., 1994). Furthermore, passive warm-up increases muscular power in the morning only, when central temperature is at its lowest (Racinais et al., 2004). This suggests that the effect of the diurnal increase in central temperature and that of a passive warm-up cannot be combined to improve muscular power, possibly due to their similar effect on muscular contractile properties (Racinais et al., 2005). It is possible that in the present study, the increase in muscle temperature following the first sprint "cancelled" out the passive warm-up effect of the diurnal increase in central temperature on subsequent sprints.

Diurnal Increase in Power Decrement

Our results showed that P_{peak} failed to be influenced by time-of-day from sprint 2 to sprint 5. As a result, there was a significant increase in P_{dec} from the morning to the evening. As previously reported (Bishop et al., 2001), P_{dec} was correlated with P_{peak} of the first sprint ($r = 0.81$, $p < 0.05$). This finding suggests that the higher P_{peak} observed during the first sprint in the evening may have contributed to the greater P_{dec} observed in the evening. From a physiological point of view, a higher P_{peak} could lead to higher metabolic disturbances, greater PCr depletion, and less subsequent PCr repletion that in turn might increase P_{dec} (Bogdanis et al., 1996; Dawson et al., 1997). If this was the case, power during subsequent sprints should be less in the evening than in the morning from the sprint 2 on. The absence of a diurnal difference both for sprints 2, 3, 4, and 5 and for the total work done suggests that rather than a lower ability to repeat sprint performance the higher P_{dec} in the evening is a mathematical consequence of the higher power output in sprint 1, but not in subsequent sprints.

Physiological Responses, Psychological Factors and Motivation

Consistent with previous research (for a review, see Atkinson and Reilly, 1996), our subjects considered themselves to be in a better mood, and to have a greater motivation, arousal, and energy before the evening test. This higher "motivation state" at rest may also have contributed to the higher P_{peak} observed during the first sprint in the evening. However, despite a better 'mood' before the evening test, there was no diurnal variation in RPE during the repeated sprint test; this is consistent with the results of Deschenes and colleagues (1998). Our results showed a higher power decrement across the RSA test which could be linked with the significant increase in $[La^-]$ from morning to evening (values post-exercise: 11 ± 3 *vs.* $13 \pm 3\,mmol/L^{-1}$, +12%). However, the absence of diurnal variation in both the decrease from P_{peak} to P_{end} for each sprint and the total work done suggest an equivalent fatigue in the morning and the evening. The increase in $[La^-]$ from morning to evening is likely due to the higher power output during first sprint in the evening rather than to a biological rhythm.

CONCLUSION

The major finding of the present study is that P_{dec} during a 5×6 sec RSA test is affected by time-of-day. It appears that muscle power is improved in the evening during the first sprint but that there was a

common end-point (fatigue) between morning and evening when the sprints are repeated. The absence of a diurnal difference for the total work done suggests that it was not that fatigue was higher in the evening, but that the increased power decrement from the first sprint is the consequence of a higher first sprint. While the explanation for the time-of-day effect on muscular power is still unknown, our data suggest that the beneficial effect of time-of-day may be limited to a single expression of muscular power, and fails to advantage performance during repeated sprints. Further research is needed to understand the effects of time-of-day on muscular power and the consequences for repeated-sprint ability.

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Chronobiology International, 22(6): 1041–1054, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500397959

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HERITABILITY OF MORNINGNESS-EVENINGNESS AND SELF-REPORT SLEEP MEASURES IN A FAMILY-BASED SAMPLE OF 521 HUTTERITES

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Individual variation in the phase and amplitude of human circadian rhythms is well known, but the impact of heritable factors on such variation is less clear. We estimated the narrow-sense heritability for selected circadian and sleep timing, quality, and duration measures among related members of the Hutterites, an endogamous, religious community ($n = 521$ participants). "Morningness-eveningness" (M/E), a stable trait reflecting circadian phase, was evaluated using the Composite Scale (CS). Subjective sleep measures were assessed using the Sleep Timing Questionnaire. Initial analyses reconfirmed the impact of age on M/E. Previously reported correlations between M/E scores and the sleep measures were also noted, demonstrating the construct validity of the questionnaires among the participants. Following corrections for age, gender, and colony of residence, significant narrow-sense heritability was noted for M/E (23%). The heritability for subjective sleep measures (related to timing, duration, and quality) were statistically significant for all but one variable, and varied between 12.4% and 29.4%. Thus, significant heritable influences on human circadian phase

Submitted August 20, 2005, Returned for revision September 2, 2005, Accepted September 2, 2005

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and subjective sleep indices can be detected through family-based studies. In view of the impact of circadian malfunction on human health, it may be worthwhile to map genetic factors impacting circadian and sleep variation.

Keywords Heritability, Morningness, Eveningness, Sleep, Hutterite, Genetics, Circadian rhythm

INTRODUCTION

Circadian variation has been documented in multiple domains of human function and can have an important impact on normal human function. Disruption of circadian rhythms has been documented in several disorders, including sleep and mood disorders (Taheri and Mignot, 2002; Hastings et al., 2003; Kripke et al., 2005; Murray et al., 2005). Therefore, it is important to understand factors that have an impact on human circadian variation. Observed phase and amplitude measured from human circadian rhythms reflect a mixture of influences, both from the endogenous circadian pacemaker and from exogenous influences. Heritable influences on circadian function are known to be present, but have not been investigated extensively. In this report, we estimate the distribution and the heritability of two sets of traits among the Hutterites, namely "morningness/eveningness" (M/E) and the self-reported timing, duration, and quality of sleep.

M/E is based on the familiar notion of dividing the world into morning people and night people; *i.e.*, into different "chronotypes." M/E variation is normally distributed in the population. Morning-type and evening-type individuals differ in the phase position of their endogenous circadian oscillator (Kerkhof and Van Dongen, 1996; Duffy et al., 2001). In addition to predicting the timing of sleep onset and offset, measures of M/E are also associated with inter-individual differences in the phase (timing) of physiological circadian rhythms in plasma cortisol levels and core body temperature, as well as behavioral rhythms related to eating, exercise, and performance (Horne and Östberg, 1976; Ishihara et al., 1987; Monk et al., 1991; Matsumoto et al., 1996; Nebel et al., 1996; Baehr et al., 2000).

A study of 285 U.S. twins suggested that genetic variability accounted for approximately 54% of the total variance in M/E; whereas, age contributed to 3% of the variance (Hur et al., 1998). Another Dutch twin study estimated heritability at 44% for M/E (Vink et al., 2001). A recent study of 888 Korean twins revealed a heritability of 43% (Dr. Yoon-Mi Hur, personal communication). Investigation of 29 families in an Amazonian river community suggested a heritability of 14% (Aguiar et al., 1991). To our knowledge, heritability estimates using larger samples of multi-generational families are unavailable for M/E.

Sleep timing, duration, and quality are obvious and important variables of interest in human circadian studies. A 2002 twin study estimated heritability for self-reported measures of sleep, including the time that the individual went to sleep and woke up, how often the individual woke up during the night, the duration of sleep and wakefulness, and how alert the individual felt upon waking and over the day. Significant heritability estimates, between 21% and 41%, were obtained (de Castro, 2002). Estimates of sleep duration from clinical interview schedules can be unreliable and polysomnographic sleep recording was not feasible for the present study. Sleep diaries could be used instead, but are cumbersome, requiring the individual to complete the diary every night for 1 or 2 wks. Instead, we elected to use the Sleep Timing Questionnaire (STQ), a simple yet reliable measure of sleep duration, timing, and quality that has been validated against subjects also completing full 2 wk sleep diaries and wrist actigraphy studies (Monk et al., 2003).

We conducted our studies among the Hutterites, a genetically homogenous religious group whose members have a relatively uniform lifestyle. The Hutterites, who were established as an Anabaptist sect in 1526 in the Tyrolean Alps (parts of modern Southern Germany, Austria, Switzerland, and Northern Italy), migrated to the USA between 1874 and 1877. Upon arrival in the U.S.A., approximately 443 individuals (101 couples) continued traditional Hutterite practices, such as communal living. These individuals, along with 3 large families that joined the Hutterites between 1883 and 1892, and a few additional individuals, constitute the ancestors of the current Hutterite community (Eaton and Mayer, 1954). The structure of the Hutterite population provides an unusual opportunity to analyze genetic factors impacting complex traits while reducing the impact of environmental factors. The Hutterites live in agrarian, largely autonomous, communities they call "colonies." Each colony has 80 to 90 individuals residing as nuclear family units that are inter-related (Martin, 1970). The residence history can be determined throughout an individual's life and is generally stable through adulthood. The presence of extensive genealogical records, reliable vital statistics, and well documented origins facilitate genetic studies among the Hutterites (Bryttan et al., 1994). Other advantages include the relatively large cooperative families and low rates of emigration and immigration. In addition, the virtual absence of substance abuse facilitates phenotypic definition.

MATERIAL AND METHODS

Participants and Consenting Procedures

Our study conformed to the guidelines of the Declaration of Helsinki as required by the Journal (Touitou et al., 2004). Participants were

recruited from Hutterite colonies in Manitoba. All members of the Hutterite community 18 yrs or older were eligible to participate. All participants provided written informed consent. In order to solicit "communal consent" prior to conducting the studies, the investigators held detailed discussions with Hutterite members in the selected colonies and explained the goals of the studies as well as their potential impact. Subsequently, permission for the study was sought from community leaders. Approval for the study was also obtained from the University of Pittsburgh Institutional Review Board. Facilitating members of the Hutterite community were included as co-investigators (SM, DG, TW).

Genealogy

We obtained a copy of the complete Hutterite genealogy from our Hutterite collaborators. The number of founders has been estimated from historical data at 91 or 150 genomes (Martin, 1970). The average coefficient of inbreeding of over 10,000 individuals considered to comprise a 1981 census population on one Hutterite kinship group was 0.0338 ± 0.0149 (Weiler et al., 1997). Our genealogy consists of detailed information on 51,312 individuals. We used a method of pruning to reduce the pedigree to 1799 essential individuals (Klei et al., 1993). Essential individuals are defined as those who have either an observation on M/E and subjective sleep or are on a pedigree path connecting two individuals with an observation.

Circadian Variables

Data were collected on M/E, sleep related-measures, as well as demographic and health-related issues using simple questionnaires. The questionnaires used for the M/E and sleep measures were considered as proxies to more invasive and time-consuming measures of sleep and circadian rhythms. The latter are clearly preferable, but were not feasible here (Frank et al., 2000; Monk et al., 1990, 2002, 2003, 2004). Participants completed the questionnaires individually in the presence of the investigators, who provided guidance as needed.

Evaluation of M/E

The Composite Scale (CS) is a validated adaptation of the Horne-Östberg scale that is used to assess M/E (Horne and Östberg, 1976; Smith et al., 1989). This self-report questionnaire gauges diurnal preference for activity and is composed of 13 items rated on a scale of 1 to 5 and can be completed in 5 to 10 min. Scores range from 13 to 55. "Morning" persons score high on this scale and are defined (in the original publication) as those with scores of 44 or more; evening persons score 22

or less. The remainder form an intermediate group (scores, 23 to 43). In the present study, the CS score was regarded as a continuous variable measuring degree of M/E.

Sleep-Related Measures: Sleep Timing Questionnaire (STQ)

We designed the STQ, a single administration self-report instrument consisting of 18 questions (Monk et al., 2003), which can be completed in 10 min. It conveniently yields standard measures of sleep, such as sleep latency, wake after sleep onset (WASO), and habitual timing of a person's sleep. STQ measures of sleep timing showed good reliability and validity, correlating well with both wrist actigraph-based and 2 wk, diary-based measures (Monk et al., 2003). We selected 7 measures from the STQ: 1) good night time (BEDTIME, in min after midnight), 2) good morning time (WAKE TIME, in min after midnight), 3) sleep time (SLPT, in min), 4) stability of BEDTIME (SBEDTIME, in min), 5) stability of WAKE TIME (SWAKE TIME, in min), 6) time to fall asleep after start of trying (FSLP, in min), and 7) wake after sleep onset (WASO, in min).

The STQ elicits information for sleep timing variables based on nights before workdays and on nights before days off (weekends). We elected to use a weighted average of the weekday and weekend measures in our analyses. Weekday and weekend measures were weighed with a factor of 5 and 2, respectively.

Stability of BEDTIME and WAKE TIME is obtained in the STQ on a categorical scale with 11 classes. The first 8 classes are based on 15 min intervals up to 2 h. Class 9 is the 2 to 3 h class, class 10 the 3 to 4 h one, and class 11 is used for more than 4 h. To obtain a continuous variable we translated each class to its mid-point in min (*e.g.*, class 2, 16-30 min was assigned the value 23). For class 11 (more than 4 h), we used 300 min.

Statistical Analyses

To determine heritability, we used the software package Solar2 with a model including fixed effects for age, sex, and colony, and random effects for individual and residual (Williams and Blangero, 1999a, 1999b; see also http://www.sfbr.org./solar/). Solar2 uses pedigree analysis, taking into account inbreeding as well as all kinships among individuals. This program only analyzes regression and binary fixed effects. Therefore, the 18 colonies in which participants resided were modeled as 17 binary regressions (the effect of the 18th colony was set to 0). The analysis for each phenotype was performed in two steps. The initial step was to screen the 19 fixed effects (age, sex, and the 17 colony effects) for significance. Subsequently, the heritability for the phenotype was estimated

using a model with the significant effects from the first step. Significance was set at $p \leq 0.10$ (the Solar2 default).

RESULTS

Demographic Features of the Participants

Although the Hutterites lead a relatively uniform lifestyle, we reasoned that daily schedules may vary between colonies (Table 1). Therefore, colony of residence was considered an important variable. Data were collected on 556 individuals residing in 26 colonies. Eight colonies were represented by fewer than 5 individuals, and data for the individuals from these colonies were therefore removed from the analysis ($n = 12$). Also edited from the dataset were 1 set of twins, 1 individual identified with chronic fatigue syndrome, 11 individuals older than 70 yrs, and 9 people who did not provide information on sleep time. These edits resulted in a dataset of 521 individuals from 18 colonies. The average number of participants per colony was 29 (range, 9 to 52). The average age of the participants was 37.3 yrs. The age distribution was even across the 18 to 50 yr range, with a drop-off in participation beyond 50 yrs. The female-to-male ratio for participants was

TABLE 1 Demographic Details

Colony	Individuals, *n*	Men, *n*	Women, *n*	Men average age (yrs)	Women average age (yrs)
1	29	9	20	36.0	36.4
2	53	22	31	33.5	39.1
3	11	4	7	32.3	33.0
4	17	4	13	49.0	40.2
5	30	9	21	34.2	41.5
6	26	5	21	32.6	34.8
7	46	17	29	38.7	35.8
8	37	18	19	36.4	36.4
9	18	5	13	34.0	34.2
10	38	17	21	34.9	35.3
11	17	3	14	42.3	38.6
12	9	1	8	40.0	44.0
13	26	6	20	34.5	42.2
14	51	24	27	37.8	40.0
15	52	23	29	36.0	36.6
16	24	4	20	26.3	38.0
17	24	8	16	43.1	37.1
18	13	2	11	27.5	38.6
Total	521	181	340	36.2	38.8

approximately 2 to 1, with substantial variation between colonies. The female participants were on average 1.6 yrs older than the males.

Distribution of Circadian Measures

The average score for M/E was 41.0 (±5.8), consistent with normative data (Table 2). The BEDTIME was on average 22:42 h (±45.4 min), while the WAKE TIME was on average 07:12 h (±39.3 min), with an average of approximately 8:30 h (±56.0 min) of sleep duration (SLPT). Approximately half the participants reported that they woke up between 06:30 and 07:00 h. On average there were about 48 (±43.6) min of variation in the time for going to bed (SBEDTIME), while in the morning the variation in time for waking up was 26 (±31.9) min (SWAKE TIME). It took the average participant approximately 22.5 (±22.3) min to fall asleep, and the average participant spent 17.6 (±26.4) min awake during the night. Table 2 also shows that the variation in the phenotypes measured in the evening (BEDTIME and SBEDTIME) is larger than those recorded for the morning variables (WAKE TIME, and SWAKE TIME).

One of the assumptions of the analysis is that the residuals are normally distributed. Plots of the data (not shown) revealed skewness for each of the measures. M/E and BEDTIME showed a longer tail than expected tail for lower values (skew left), while all others showed a longer than expected tail for higher values (skewed right) (see also Table 2). To counter this we used a base 10 log-transformation for the variables of SBEDTIME, SWAKE TIME, FSLP, and WASO.

TABLE 2 Distribution of Morningness/Eveningness And Sleep Measures

Phenotype	Mean	SD	Minimum	Q1	Median	Q3	Maximum
M/E	41.0	5.8	19.0	38.0	42.0	46.0	46.0
BEDTIME	1362.5	45.4	1187.0	1335.0	1359.0	1389.0	1539.0
WAKE TIME	432.8	39.3	240.0	420.0	420.0	437.0	716.0
SLPT	510.3	56.0	351.0	480.0	508.0	538.0	795.0
SBEDTIME	48.3	43.6	8.0	23.0	38.0	53.0	300.0
SWAKE TIME	26.4	31.9	8.0	8.0	19.0	27.0	217.0
FSLP	22.5	22.3	1.0	10.0	15.0	30.0	210.0
WASO	17.6	26.4	0.0	5.0	10.0	20.0	240.0

M/E: Morningness-Eveningness score (from Composite scale); BEDTIME: Good night time (min); WAKE TIME: Good morning time (min); SLPT: Sleeping time (duration of time in bed in min); SBEDTIME: Stability of bedtime (min); SWAKE TIME: Stability of wake time (min); FSLP: Time to fall asleep after starting to try (min); WASO: Time awake after sleep onset (min); Q1, Q3: First and third quartiles, respectively; All times are recorded as time since midnight (00:00 h) in min. SD = Standard deviation.

Correlations Between Circadian Measures

There were predictably strong correlations between SLPT and BEDTIME (−0.72) and between SLPT and WAKE TIME (0.60). Interestingly, the correlation between BEDTIME and WAKE TIME was much lower (0.13), indicating that sleep duration (or, more accurately, duration of time in bed) was not constant across subjects, but instead showed important inter-individual variability. Consistent with the communal agrarian work performed by the majority of the participants, a large fraction reported waking up at approximately the same time. There was strong correlation (0.57) between the two stability measures. Thus, individuals who tend to go to bed each night at the same time also wake up at the same time each morning, while those who have a variable BEDTIME also tend to have a variable WAKE TIME. There was a moderate correlation (0.46) between FSLP and WASO, suggesting that individuals who found it difficult to fall asleep also spent a lot of time awake at night (*i.e.*, those with poor sleep quality had so in both variables) (Table 3).

Consistent with the definition of M/E, "morning" type persons tended to go to bed early (correlation between M/E score and BEDTIME: −0.33,) and to wake up early (correlation between M/E score and WAKE TIME: −0.37). Morning type persons also have a tendency to go to bed and wake up consistently at about the same time (correlation is −0.25 with both SBEDTIME and SWAKE TIME), and tend to have an easier time falling asleep (correlation is −0.11 with FSLP). Thus, morning type persons appeared to be more likely than evening persons to have predictable and regular sleep habits. They did not sleep longer or spend less time awake during the night than evening persons.

TABLE 3 Correlations Between Circadian Phenotypes

	M/E	BEDTIME	WAKE TIME	SLPT	SBEDTIME	SWAKE TIME	FSLP
BEDTIME	−0.33						
WAKE TIME	−0.37	0.13					
SLPT		−0.72	0.60				
SBEDTIME	−0.25	0.11	0.14				
SWAKE TIME	−0.25		0.10		0.57		
FSLP	−0.11	−0.12		0.16	0.22	0.17	
WASO		−0.21		0.18	0.13		0.46

Only correlations significantly different from zero are listed ($p < 0.05$).

M/E: Morningness-Eveningness score (from Composite scale); BEDTIME: Good night time (min); WAKE TIME: Good morning time (min); SLPT: Sleeping time (duration of time in bed in min); SBEDTIME: Stability of bedtime (min SBEDTIME); SWAKE TIME: Stability of wake time (min SWAKE TIME); FSLP: Time to fall asleep after starting to try (min); WASO: Time awake after sleep onset (min).

Impact of Age, Gender and Colony of Residence on Circadian Measures

Age

There was a significant effect of age ($p < 0.05$) for all variables except the two stability measures (SBEDTIME and SWAKE TIME, Table 4). Older participants tended to go to bed earlier (1 ± 0.136 min/yr, BEDTIME), wake up earlier (0.3 ± 0.126 min/yr, WAKE TIME), and effectively spent 0.7 (± 0.169) more min/yr of age of time in bed at

TABLE 4 Parameter Estimates

	M/E			BEDTIME		
	Estimate	SE	*p* Value	Estimate	SE	*p* Value
Standard deviation	5.61	0.18		41.3	1.3	
Heritability	0.237	0.092	<0.001	0.095	0.096	0.285
Gender	0.848	0.525	0.096			>0.100
Age	0.110	0.018	<0.001	−0.963	0.136	<0.001
Significant colonies*	7, 8, 10, 15, 17			2, 5, 6, 7, 10, 11, 14		
	WAKE TIME			SLPT		
Standard deviation	38.44	1.2		53.6	1.7	
Heritability	0.124	0.060	0.009	0.259	0.080	<0.001
Gender	−10.2	3.6	0.001			>0.100
Age	−0.314	0.126	0.010	0.613	0.169	<0.001
Significant colonies*	7, 12			2, 7, 10, 15		
	$\log_{10}$ SBEDTIME			$\log_{10}$ SWAKE TIME		
Standard deviation	0.333	0.011		0.335	0.011	
Heritability	0.294	0.097	<0.001	0.137	0.078	0.022
Gender			>0.100	−0.132	0.033	<0.001
Age	−0.002	−0.001	0.089			>0.100
Significant colonies*	1			2, 10, 15		
	$\log_{10}$ FSLP			$\log_{10}$ WASO		
Standard deviation	0.344	0.011		0.514	0.016	
Heritability	0.163	0.093	0.021	0.201	0.084	<0.001
Sex	0.054	0.032	0.071			>0.100
Age	0.003	0.001	0.002	0.016	0.002	<0.001
Significant colonies	14					

M/E: Morningness-Eveningness score (from Composite scale); BEDTIME: Good night time (min); WAKE TIME: Good morning time (min); SLPT: Sleeping time (duration of time in bed in min); SBEDTIME: Stability of bedtime (min SBEDTIME); SWAKE TIME: Stability of wake time (min SWAKE TIME); FSLP: Time to fall asleep after starting to try (min); WASO: Time awake after sleep onset (min).

*Significance based on $p \leq 0.100$ when screened.

night (SLPT). Older people also tended to evaluate themselves more as morning people. The effect of age was 0.11 (±0.018) points on the M/E scale/yr of age. Finally, there were small but significant effects on the time it takes for a person to fall asleep and the time spent awake at night. These values were 0.003 (±0.001)/yr of age on the log scale for FSLP and 0.016 (±0.002) on the log scale for WASO.

Gender

The effect of gender was significant ($p < 0.05$) for only two of the variables (WAKE TIME and SWAKE TIME). The estimate of the parameter for WAKE TIME suggested that women tended to wake up 10 (±3.6) min earlier than men and that they also tended to show less variability in their good morning time. Since no significant differences were noted for the majority of variables, the data for both genders were combined when estimating heritability.

Colony

There were significant differences across colonies for less than half the variables ($p < 0.10$). These differences were affected by the number of participants/colony, with the largest colonies showing significant effects (data not shown).

Heritability Estimates

Narrow sense heritability estimates were significant for all but one of the phenotypes (BEDTIME). The heritability estimate for M/E was 23.7% ($p < 0.001$). Among the sleep quality measures, the highest estimates of heritability were observed for SBEDTIME on the log transformed scale (29.4%), duration in bed (SLPT, 25.9%), and time awake after sleep onset on the transformed sale (WASO, 20.1%). The p values for these 3 estimates were highly significant ($p < 0.001$). These heritability estimates were similar to the one observed for M/E. Estimates for the heritability of WAKE TIME and SWAKE TIME on the log scale were similar at 12.4% ($p = 0.009$) and 13.7% ($p = 0.022$), respectively. The heritability estimate for the time to fall asleep (FLSP) on the log scale was 16.3% ($p = 0.021$). The only sleep quality measure that did not show a significant estimated heritability was BEDTIME.

DISCUSSION

We used simple pen-and-paper questionnaires to evaluate M/E and sleep characteristics in a large, unselected Hutterite sample. As would be

expected of an agrarian community, the average M/E score suggested a tendency toward morningness. Previously reported correlations between age and the circadian measures were also detected (Carrier et al., 1997). As one would predict, significant correlations were detected between M/E and BEDTIME, as well as between M/E and WAKE TIME. Our analyses suggested that morning type persons tended to have more regular lifestyles, as we have reported earlier in a very different sample (Monk et al., 2004). The reported correlations suggest construct validity for the questionnaires among the Hutterites. Skewness was detected for some measures and may be attributed to the restricted age range. Our sample included more women than men, but significant gender differences were only detected for two circadian measures (BEDTIME and log transformed SBEDTIME).

Following appropriate adjustments for age, gender, and colony of residence, we detected significant heritability for M/E, as well as for all sleep measures except BEDTIME. Our estimate for heritability of M/E (23%) was much lower than the 44% value reported following a survey of healthy Dutch twins (Vink et al., 2001). Heritability values from twin data are not only based on the additive genetic variance but also on all other variation that they have in common. This includes part of the dominance variance, and possibly some permanent environment effects (the dominance variation estimates for the Dutch samples was 15% to 16%). Thus, twin data yield estimates of the broad-sense heritability. Our design and analysis, based on all known relationship information within families, minimizes (but may not eliminate) confounding with these additional variance terms. As a result, the narrow-sense heritability obtained from our study might give a better estimate of the true proportion of the variance explained by transmissible additive gene effects.

There are other plausible explanations for the lower than expected heritabilities. One might be our use of self-report questionnaires. Even though there is good agreement between these questionnaires and the data collected through other means, the accuracy of self-reported data might be lower. This could increase the non-genetic component of the variability and result in a decrease in the heritability estimates. However, such an explanation would require that the accuracy of self reporting differed between the Hutterites and participants in the earlier studies. We do not have any evidence for such differences. The nature of the Hutterite lifestyle might also provide an explanation for the lower heritability estimates. The Hutterites have a relatively uniform temporal environment, stemming from their communal lifestyle and relatively fixed schedule. For instance, communal breakfast is served at approximately the same time for all individuals and may explain the relatively uniform WAKE TIME noted here. Arguably, even measures based on *preference*, such as M/E, may be influenced by the communal influences. Similar conclusions

were reached in a study of a population indigenous to the Amazon river basin (Aguiar et al., 1991). In that study, an adaptation of the Horne-Östberg scale was used to estimate M/E among members of 29 families. The majority of the members (95.35%) were classified as morning types. The heritability was estimated at 14% and the relatively low estimate was attributed to "masking" effects of socio-cultural factors.

It is well known that environmental restrictions can have a significant impact on heritability estimates. For example, a twin study of regular tobacco use (RTU) in Sweden revealed different rates of heritability among women, depending on the year of birth. Among women born before 1925, rates of RTU were low and twin resemblance was evidently environmental in origin. In later cohorts, rates of RTU in women increased substantially, as did heritability. The results suggest that as social restrictions on female tobacco use relaxed over time, heritable influences increased in importance (Kendler et al., 2000). Research on farm animals has also revealed that a restrictive environment can have a substantial impact on heritability estimates (Boldman and Freeman, 1990; Zwald et al., 2005). It was suggested that the restricted environment did not facilitate full expression of genetic differences, leading to a reduction in genetic variance and lower heritability estimates. In the same vein, the relatively uniform and restricted Hutterite lifestyle may result in lower than expected heritability estimates. A final explanation of the smaller heritability estimates could be the relatively small number of founding genomes represented in the Hutterite population.

In conclusion, we investigated the distribution and heritability of traits related to circadian variation among Hutterite individuals. Predictable correlations between measures of M/E and sleep characteristics were noted, and the previously reported correlations with age were documented. Significant narrow sense heritability was noted for all the circadian phenotypes evaluated, with the exception of BEDTIME. The heritability was lower than expected, and may be attributable to the relatively uniform lifestyle among the Hutterites.

ACKNOWLEDGMENTS

We thank all our study participants. Comments from Dr. Bernie Devlin and Dr Yoon-Mi Hur are gratefully acknowledged. We thank Ms. Karol Rosengarth for help with manuscript preparation. Supported in part by grants from the National Institutes of Health (MH63420, MH56242 and MH66263 to VLN; AG13396, and AG020677 to THM), and NASA (NAG9-1234 and NNJ04HF76G to THM).

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Chronobiology International, 22(6): 1055–1068, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398007

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ASSOCIATION BETWEEN MORINGNESS-EVENINGNESS PREFERENCE AND MENTAL/PHYSICAL PREMENSTRUAL SYMPTOMS IN JAPANESE FEMALES 12 TO 31 YEARS OF AGE

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This study investigates the relationship between circadian typology, *i.e.*, morningness-eveningness (M-E) preference, and the occurrence and severity of premenstrual mental and physical symptoms among 154 young Japanese female university students (range, 18 to 31 yrs; mean ± S.D., 20.69 ± 3.69 yrs) and 417 junior high school students (range, 12 to 15 yrs; mean ± S.D., 14.29 ± 0.67 yrs) living in an urban or suburban area of Kochi prefecture. Female university students experienced melancholy mood more frequently than did males, and the female university students who frequently became melancholy were more evening-typed than those who did not experience melancholy. Female university students who experienced frequent fluctuations in mood and/or menstrual pain were more evening-typed than those who were not so affected. M-E preference of junior high school students was not correlated with stability of mood or frequency of menstrual pain. In urban areas, however junior high school students who had very stable menstrual cycles were significantly more morning-typed than those whose menstrual cycles were not stable. In suburban areas, the bedtimes of female junior high students who had stable menstrual cycles were significantly earlier than those whose menstrual cycle duration was not stable. A physiological relationship between the circadian system, M-E, and attributes of the menstrual cycle seems to be present in adolescent female Japanese junior high school students.

Keywords Morningness-eveningness preference, Circadian rhythm, Menstrual cycle, Premenstrual syndrome, Japanese students

INTRODUCTION

Only a small number of studies have been conducted on the morningness-eveningness (M-E) preferences of children (Bearpark and Michie, 1987;

Submitted June 20, 2005, Returned for revision September 15, 2005, Accepted September 23, 2005

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Carskadon et al., 1983; Ishihara et al., 1990; Harada and Takeuchi, 2001; Kim et al., 2002). M-E is believed to be determined both by biological/genetic and psychosocial factors, including sex (Kerkhof, 1985; Vink et al., 2001; Adan, 2002; Kim et al., 2002). Carskadon and Natale (1983) reported the phase delay in the sleep and wake-up times in children are explainable by psychosocial (*e.g.*, birth order and peer group) or biological (*e.g.*, puberty) factors. In this study of pubertal sixth grade boys and girls, biological, rather than psychosocial, factors were found to be related to the phase delay of sleep and wake up times. Takeuchi and colleagues (2003) found that young Japanese girls aged 12 to 15 yrs of age who had been menstruating for more than 1 yr after menarche were more evening-typed than those who had not yet begun menstruating. Bearpark and Michie (1987) and Harada and Takeuchi (2001), in studies of children aged 10 to 17 yrs of age, found that M-E scores significantly decreased with age, meaning greater evening preference. Harada and Takeuchi (2001) reported that rapid shifting to eveningness between fifth grade (10 to 11 yrs old) elementary school and first grade (12 to 13 yrs old) junior high school Japanese children. Ishihara and coworkers (1990) reported a similar finding in Japanese females aged 9 to 15 yrs, *i.e.*, change in preference toward eveningness with advancing grades in school.

Positive association between evening-type preference and depressive mood has been previously described in adults (Chelminski et al., 1999; Drennan et al., 1991) and adolescents (Giannotti and Cortesi, 2002; Harada, 2004). Several reports also have shown that de-synchronization of the circadian clock system and the social time schedule is often accompanied by depressed mood (Weitzman et al., 1981; Thorpy et al., 1988). Fukuda and Hozumi (1987) reported that Japanese high school students who refused to go to school is more likely to commit "domestic violence" against their mother during an epoch when the rest-activity rhythm is disrupted than during other times when the circadian sleep-wake rhythm is clearly expressed. Fukuda and Ishihara (2000) also showed that irregularity of the circadian phase of night sleep is accompanied by lassitude and depressed mood in Japanese students. One of our recent studies found that the M-E preference of Japanese students is currently changing, with a greater proportion being evening-types with later (delayed) bedtimes (Harada and Takeuchi, 2001), and this shift to evening-type behavior has prompted concern about the possibility of an increased prevalence of depressive mood among Japanese students (Harada, 2004).

Previous research shows that Japanese female junior high school students became melancholy more frequently than male students, and that females, who are prone to melancholy mood, are more evening-typed than those who are not prone to become melancholy (Takeuchi et al., 2002). If depressed-mood accompanied by evening-type overlaps with the premenstrual syndrome (PMS) (Parry et al., 1990), it is plausible that

the mental and physical symptoms of PMS may become severe. We wondered if there exits a relationship between circadian typology and the occurrence and severity of PMS in young Japanese girls and women. There have been few studies on this subject to date. Thus, the present study investigated whether there is a correlation between M-E preference and mood in Japanese junior high school students, and whether there is a relationship between M-E preference and PMS physical and mental symptoms in young adolescent Japanese girls aged 12 to 15 yrs and in more mature Japanese young women aged 18 to 22 yrs.

PARTICIPANTS AND METHODS

Morningness-Eveningness Questionnaire

A Japanese MEQ version was used (Takeuchi et al., 2001). The questionnaire was translated from the English version and modified slightly for students (Harada and Takeuchi, 2001), based on the MEQ of Torsvall and Åkerstedt (1980), originally constructed for use on samples of workers. Three of the 7 questions included in the MEQ pertain to sleep onset timing in the evening, 3 to sleep offset timing in the morning, and 1 to peak timing of activity during the daytime. Each question allows for choice (scored from 1 to 4), from evening- to morning-type. The M-E score is the summation of the 7 answers. The minimal possible score (extreme evening-type) is 7, and the maximal possible score (extreme morning-type) is 28. The original questionnaire, including the 4 questions on mental or physical symptoms, is shown in Table 1.

Subjects

The MEQ and the original questionnaire were administrated to 417 female junior high school students aged 12 to 15 yrs in Kochi Prefecture (33°N) and 252 (154 women, 98 men) university students aged 18 to 31

TABLE 1 Questions on physical and mental symptoms in relation with menstrual cycle

Q1: How frequently is your mood depressed?
A1: (1) Always, (2) Often, (3) Sometimes, (4) Rare or Not al all
Q2: How frequently does the period of your menstrual cycle change longer and/or shorter?
A2: (1) Always, (2) Often, (3) Sometimes, (4) Rare or Not at all"
Q3: How frequently do you feel fluctuating to positive and/or negative in mood before or during menstruation?
A3: (1) Always, (2) Often, (3) Sometimes, (4) Rare or not at all
Q4: How frequently do you feel belly ache during menstruation?
A4: (1) At every menstruation, (2) Often, (3) Sometimes, (4) Rare or Not at all

yrs attending Kochi University. Among the 417 junior high school students, 215 were attending a junior high school in an urban area in Kochi city, and 202 were residing in a suburban area either in Nankoku city (33°N) or a mountain area of Reihoku district. The study was conducted between July and December 2001. We were unable to ask the female participants whether or not they used birth control pills or hormone patch medications. However, most young Japanese females are more likely to depend on other means of birth control, such as condoms. A separate survey of 11 females, 20 to 28 yrs of age, residing in Kochi revealed no use of birth control pills or patch medications. Moreover, more than 70% of the females aged 18 to 20 yrs recruited for our studies did not use birth control. Thus, we feel that the majority of the participants in the study reported here were not using non-birth control pill or patch medications.

Questions Pertaining to PMS and Menstrual Cycle Stability

The stability *vs.* instability of the menstrual cycle was self-assessed and self-reported by the female participants. The following questions (Q) were asked of the subjects to ascertain answers (A) on the stability of the menstrual cycle

Q: We are asking about the frequency of the change in the period of menstrual cycle (the shifting of the predicted starting-day of menstruation). Please select and check the most appropriate answer of the several choices that follow.

A: (1): The period of my menstrual cycle is very stable (no shifting of predicted starting-day)

(2): The period of my menstrual cycle is relatively stable (predicted starting-day is sometimes shifted)

(3): The period of my menstrual cycle is relatively unstable (predicted starting-day is often shifted)

(4): The period of my menstrual cycle is completely unstable (starting day unpredicted)

Another series of "Qs & As" addressed premenstrual mental symptoms of the female participants as follows.

Q1: During the one week before menstruation and menstruation period, how frequently do you feel any mental symptom?

A1: (1) every time (always)

(2) many times (often)

(3) sometimes

(4) rarely

(5) not at all

Q2: During which of premenstrual week and menstruation period, do more menstrual symptoms appear?

A2: (1) more in premenstrual week

(2) more in menstruation period

(3) the same (no-difference)

Q3: What kind of mental symptoms do you have? Please mark all the selective symptoms you feel.

A3: (1) irritated

(2): angry

(3): depressed or anxious

(4): excited

(5): tearful

(6): manic,

(7): highly variable between high and depressed moods.

The following are "Qs & As" on a menstrual pain.

Q1: How frequently do you feel pain in the uterus or around the waist during menstruation?

A1: (1) every time

(2) occasionally

(3) sometimes

(4) rarely or never

Q2: How severe is your pain during menstruation?

A2: (1) not so severe

(2) relatively severe

(3) severe

(4) very severe (pain medication desired/needed)

"Q & A" on daily depression is as follows.

Q: How frequently do you feel depressed on usual days?

A: (1) often

(2) sometimes

(3) rarely

(4) not at all

Statistical Analyses

Data were statistically analyzed by χ^2 tests, Mann-Whitney U-test and Kruskal-Wallis test using SPSS statistical analyzing software for the personal computer. M-E scores were expressed as means ± 1 standard deviation (Mean $\pm$ SD).

Procedure

The study followed the guidelines established by the Journal for the conduct of research on human subjects (Touitou et al., 2004). We carefully explained the concepts and purpose of the study in writing and orally to teachers and students, and to parents, in the case of young junior high students: "Questionnaires are in completely unregistered manner and answers to them will be used only for academic purposes." After the explanation, all the students and parents agreed to participate in the research study survey, which was administered during the school day while the students were in their classrooms.

RESULTS

M-E Preference and Mood in University Students

There was no significant gender difference in M-E preference score at the time of the conduct of this study in 2001 (men: 14.72 ± 3.18 *vs.* women: 14.54 ± 3.43) (Mann-Whitney U-test: $Z = -0.870$, $p = 0.384$). However, females subjects were found to admit to melancholy more frequently than male subjects (χ^2 test: χ^2 cal $= 9.38$, $p = 0.025$) as shown in Table 2. To analyze the relationship between the frequency of depressed mood and M-E score, the two response options for Question 1: "Rare or Not at all" and "Sometimes" were combined to form Group 1, while the options of "Often" and "Always" were combined to form Group 2 Figure 1. There was no significant difference in M-E preference between the male subjects of Group 1 (13.95 ± 3.01) and Group 2 (14.18 ± 3.33) (U-test: $Z = -0.132$,

TABLE 2 Distribution of answer of Japanese students aged 18 to 31 yrs to Question: "Do you have depressed mood frequently?"[a]

	1. Always	2. Often	3. Sometime	4. Rarely or No	Total
Man	19 (19.39)	29 (29.52)	35 (35.71)	15 (15.31)	98 (100.0)
Woman	41 (26.62)	49 (31.82)	57 (37.01)	7 (04.55)	154 (100.0)
Total	60 (23.81)	78 (30.95)	92 (36.51)	22 (08.73)	252 (100.0)

[a]Values expressed as *n*(%).

$p = 0.895$). In contrast, a statistically significant difference in M-E preference was detected between the females of the two different groups. As depicted in Figure 1, the female subjects of Group 2 who frequently felt melancholy were significantly more evening-typed (13.92 ± 3.28) than those of Group 1 (15.42 ± 3.47) (U test: $Z = -2.689$, $p = 0.007$).

M-E Preference and Mental and Physical Symptoms Related to Menstrual Cycle

Junior High School Students

Students living in the urban area were more evening-typed (13.21 ± 3.91) (Mann-Whitney's U-test: $Z = -4.28$, $p < 0.001$) and went to bed later (24.23 ± 1.22 h) (Mann-Whitney's U-test: $Z = -4.48$, $p < 0.001$) than ones living in the suburban area (14.77 ± 3.36 and 23.66 ± 1.16 h). Moreover, the menstrual cycles of the female junior high female students living in the suburban area were more stable than those in students living in the urban area (χ^2 test: $\chi^2 = 7.97$, $df = 2$,

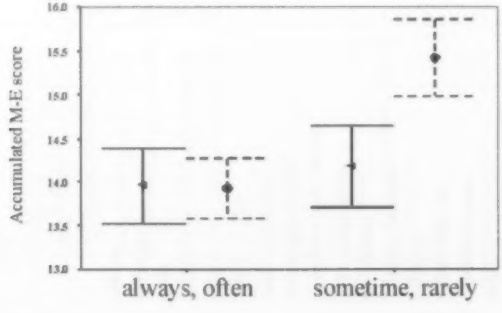

FIGURE 1 M-E preference and frequency of feeling depressed in routine life by Japanese university students aged 18 to 33 yrs. Group 1: "sometimes, rarely, not", Group 2: "always, often", –▲–: male students, –●–: female students (mean ± S.E.).

$p = 0.046$). However, as shown in Figure 2, the urban and suburban students whose menstrual cycles were stable (Stable Group of Question 2: "Rare or Not at all" and "Sometimes" unstable) were more morning-typed than those whose menstrual cycles were unstable (Unstable Group: "Often" and Always" unstable) (Mann-Whitney's U-test: $z = -2.02$, $p = 0.043$). As shown in Figure 3, junior high school students who had a stable menstrual cycle went to bed earlier than those who had an unstable cycle (Kruskal-Wallis test: $\chi^2 = 7.13$, $df = 3$, $p = 0.067$), and female students in the "Unstable Group" went to bed later those of the "Stable Group" (Mann-Whitney test: $Z = -2.20$, $p = 0.027$). Urban students whose menstrual cycle was unstable expressed fatigue more frequently (χ^2 test: $\chi^2 = 9.20$, $df = 3$, $p = 0.026$) and felt anger more frequently triggered even by slight provocation (χ^2 test: $\chi^2 = 11.46$, $df = 3$, $p = 0.009$) than urban area students whose menstrual cycle was stable.

University Students

There was no significant relationship between M-E preference and stability of the menstrual cycle (Kruskal-Wallis test, $\chi^2 = 3.31$, $p = 0.346$). However, as shown in Figure 4, female students who frequently became gloomy before or during menstruation tended to be more evening-typed than those who did not frequently become gloomy (Kruscal-Wallis test: $\chi^2 = 9.38$, $p = 0.025$). Moreover, university female students who easily became gloomy in routine life were depressed more frequently during or before menstruation (χ^2test: χ^2cal = 14.82, $p = 0.005$) and experienced menstrual pain more frequently (χ^2test: χ^2cal = 7.98, $p = 0.04$) than

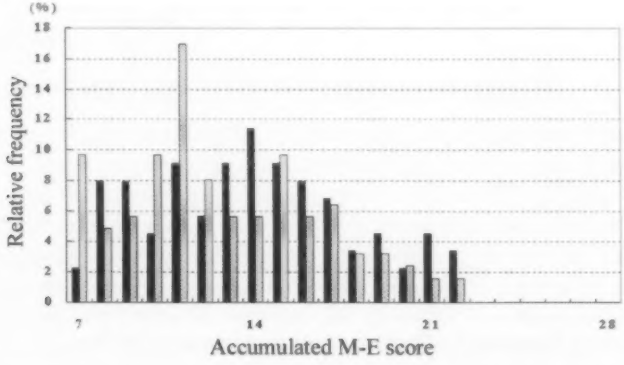

FIGURE 2 Frequency of the stability of the menstrual cycle and M-E preference of female Japanese junior high school students aged 13 to 15 yrs. ■: "stable group" in the Question 2, ▨: "unstable group"

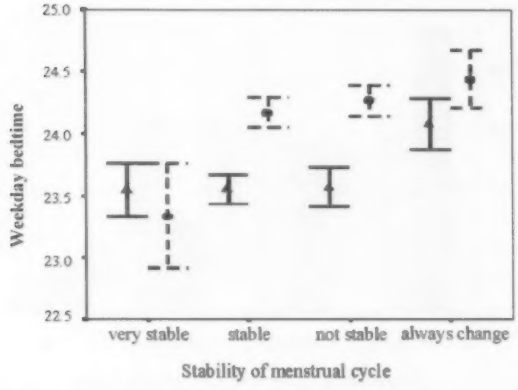

FIGURE 3 Relationship between the stability of menstrual cycle and weekday bedtime of female Japanese junior high school students aged 13 to 15 yrs. –▲–: suburban area students, –●–: urban area students (mean ± S.E.)

those who were less likely to become gloomy in routine life (Figure 5). As shown in Figure 6, female university students who frequently experienced menstrual pain more were more evening-typed than those who did not (Kruskal-Wallis test: $\chi^2 = 11.98$, $p = 0.007$). Female university students who felt gloomy "Always" or "Often" under routine conditions of life felt menstrual pain during or before menstruation more severely than those

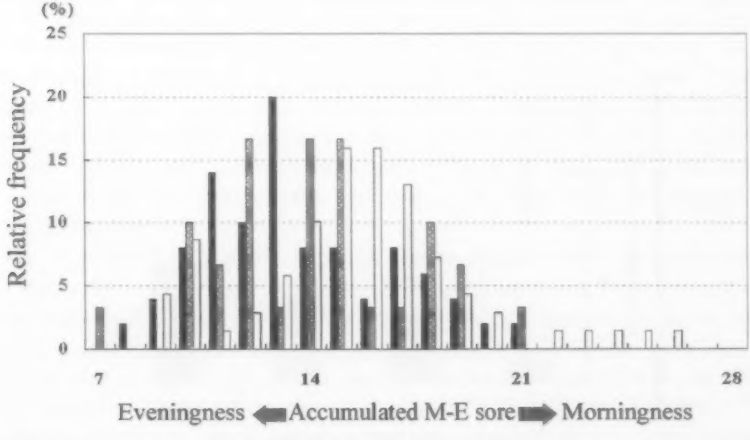

FIGURE 4 Relationship between the frequency of experiencing mood fluctuations before or during menstruation and M-E preference of female Japanese university students aged 18 to 31 yrs. ■: "always or often" experience fluctuation in mood, ▨: "sometimes", □: "rarely or not at all" as answer to Question 1.

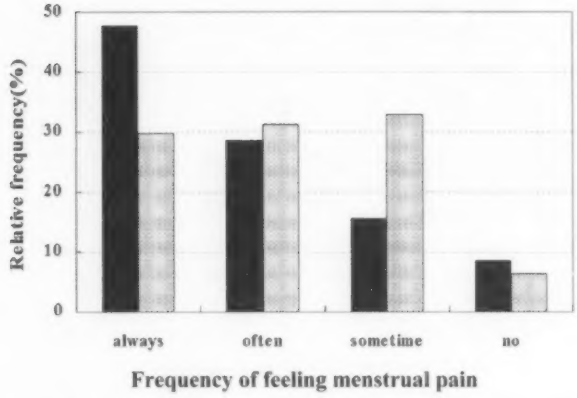

FIGURE 5 Relationship between the frequency of experiencing depressed mood in routine life and menstrual pain of female Japanese students aged 18 to 31 yrs. ■: "always or often" experience mood fluctuation, ▨: "sometimes," "rarely or not at all" as answer to Question 1.

who felt gloomy only "Sometimes," "Rare," or "Not at all" (χ^2 test: $\chi^2 = 7.98$, $p = 0.04$) (Figure 7).

DISCUSSION

In our recent study on junior high school students living in the suburban area of Nankoku City and Reihoku Disctict (33°N), we found no

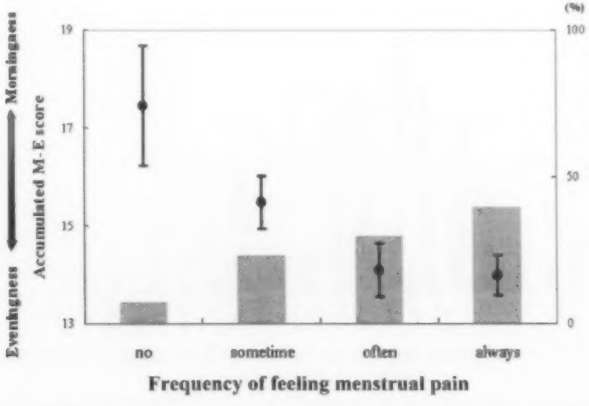

FIGURE 6 Relationship between the frequency of feeling menstrual pain and M-E preference of Japanese female students aged 18 to 31 yrs. –●–: mean ± S.E. of M-E score, □: relative frequency of the number of students (%)

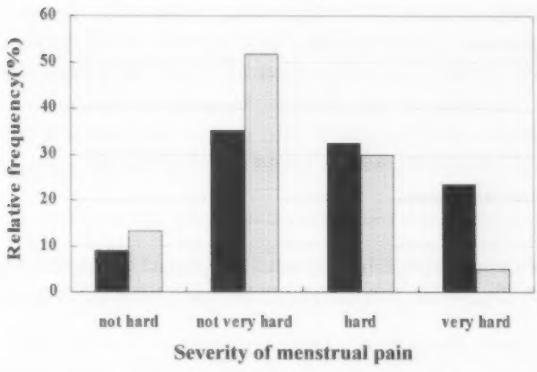

FIGURE 7 Relationship between the frequency of experiencing depressed mood in routine life menstruation and severity of menstrual pain of female Japanese students aged 18 to 31 yrs. ■: "always or often" feeling the fluctuation in mood, ▨: "sometimes," "rarely or not at all" as answer to Question 1.

significant relationship between the stability of the menstrual cycle and M-E preference (Takeuchi et al., 2003).

M-E Preference and Mood in University Students

To date, there have been several studies on the relationship between M-E preference and selected psychological variables. Levy (1985) found evening types to be more pessimistic than morning types, and Chelminski and colleagues (1999) found negative correlations between the Horne and Östberg questionnaire score and responses on the 3 depression scales of BDI (Beck Depression Inventory), GDS-SF (Geriatric Depression Scale-Short Form, 15-item), and CESD (Center for Epidemiological Studies Depression Scale). Tankova and coworkers (1994), however, in their review of studies that examined the relationship between circadian typology (M-E) and other individual difference variables, concluded that neurotism and psychotism are not associated with "morningness-eveningness" score.

The results of this study support the significant correlation between M-E score and pessimism or depression as shown by Levy (1985) and Chelminsky and colleagues (1999). Evening-typed female students more frequently felt depressed than morning-typed female students at ages 12 to 15 yrs and 18 to 31 yrs, while there was no such correlation between M-E preference and mood in male students (Takeuchi et al., 2002). This correlation between M-E preference and mood is common among Japanese children and students aged up to 22 yrs (Harada, 2004). Perhaps, psychiatric conditions are more easily affected by the circadian system in females rather than in males, and/or females are more willing

to admit depressive mood. The depressing effect of a circadian system disorder, "endogenous desynchronization" of two circadian oscillators could also play a role in the cases reported in other investigations (Weitzman et al., 1981; Thorpy et al, 1988). Moreover, hormonal fluctuations during the menstrual cycle might also induce depression as a component of PMS in female students, which could overlap the depression induced by circadian system disorder.

M-E Preference and Mental and Physical Symptoms of the Menstrual Cycle in Junior High School and University Students

Morning-typed female students attending junior high school and who reside in urban Kochi city had more stable menstrual cycles than evening-typed students, while there was no significant correlation of M-E preference with menstrual stability in the suburban area (Takeuchi et al., 2003). This result is inconsistent with the findings of our recent study on junior high school students living in the suburban area of Nankoku City and Reihoku Disctict (33°N), in which we found no significant relationship between the stability of menstrual cycle and M-E preference (Takeuchi et al., 2003). The different results between urban and suburban areas found in this study might be caused by the lower M-E scores (evening-typed) of the urban area students comparised to the scores of students living in the suburban area (Harada and Takeuchi, 2001; Fukuda and Hozumi, 1987). Change to evening-type, and later bedtime, may in some way contribute, directly or indirectly, to the instability of the menstrual cycle during the initial years of reproductive maturation in Japanese junior high school students who show rapid shifting to the evening-type (Takeuchi et al., 2003; Mary et al., 1993). In female university students who have already matured, there was no significant correlation between M-E preference and menstrual cycle stability. However, female university students who frequently felt fluctuating moods accompanying menstruation, or frequently felt menstrual pain, were more evening-typed than students who were not so affected. We speculate that depressed mood accompanied by evening-typology might lower the threshold for triggering fluctuation in mood during or before menstruation as well as menstrual pain. Perhaps, there might also be a relationship between the circadian system controlling the sleep-wake cycle and the circamensual (about monthly) system controlling the menstrual cycle; this awaits further investigation. In hamsters, the free-running period of the activity rhythm has been shown to shorten by the application of estrogens (Morin et al., 1977). Moreover, junior high school students beyond their first year after menarche have been found to be more evening-typed than those who were in their first year of menarche or those who had not yet begun menstruation (Takeuchi

et al., 2003). It is as yet unknown whether endocrine signals associated with the menstrual cycle might be a source of time cues that modify important characteristics of the female circadian system.

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Chronobiology International, 22(6): 1069–1092, (2005)
Copyright © 2005 Taylor & Francis LLC
ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398023

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FOOD INTAKE IN HEALTHY YOUNG ADULTS: EFFECTS OF TIME PRESSURE AND SOCIAL FACTORS

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Some factors influencing food intake and subjective responses to meals were assessed in 2 groups ($n = 40$ and $n = 36$) of healthy university students. Both groups were studied for 6 days and included both "structured" and "unstructured" times. A questionnaire was completed by all subjects at 3 h intervals while awake. The questionnaires asked the subjects to state the factors that led them to choose to eat or not to eat a meal in the previous 3 h. If they ate a meal, they were required to describe the type of meal eaten and their responses to it—their hunger before it, their enjoyment of the meal itself, and their degree of satisfaction afterwards. Subjects were also asked to describe the type of meal that they would like to have eaten (the desired meal) in the absence of any restraints due to time pressure, cost, and so on. In the first group, 3 "structured" (working) and 3 "unstructured" (rest) days were chosen. Consistant with our previous studies, structured days, as compared to unstructured days, were associated with smaller meals and less positive subjective responses to them. Also, the meals that were eaten were often smaller than those that were desired, or were even missed altogether, due to time pressure. Not only were the meals eaten on unstructured days larger and rated, to by the subjects more positively, but also there was an additional positive effect if the meal played a social role. In the second group, 6 days were chosen, during which there were structured and unstructured 3 h periods. Many of the findings (with regard to reasons for eating or not eating a meal, and the effect of meal size upon subjective responses to it, for example) were the same as in the first group. However, the effect of structured *vs.* unstructured 3 h periods was significantly less marked than the effect of structured *vs.* unstructured days that had been found in the first group, and effects due to social factors and time pressure were less reliably present. The results indicate that food intake is affected by whether the whole or only part of the day is "structured" or "unstructured." These findings might be relevant to some problems faced by the workforce, in general, and by night workers, in particular.

Keywords Appetite, Shift work, Meals, Lifestyle, Circadian rhythm

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INTRODUCTION

In one our previous studies (Waterhouse et al., 2004), the choice of food eaten at mealtimes was investigated using a forced internal desynchronization protocol. In this protocol, the subjects were required to live on 27 h "days," the times at which "breakfast," "lunch," and "evening meal" were eaten being shifted across all phases of the "body clock" (as assessed from the endogenous component of the circadian rhythm of core temperature). The choice of food, the reasons for making this choice, and the subjective responses to the meal (hunger before the meal, enjoyment during it, and satiety afterwards) were investigated by the "Food Intake Questionnaire" completed at regular intervals throughout waking. These aspects of food intake were the same during the forced desynchronization schedule as during control days, even though the meals were sometimes eaten in the middle of the "night," according to body time as judged by the circadian rhythm of core temperature. Also, after real (Waterhouse et al., 2000; Waterhouse et al., 2003b; 2005c) or simulated (Waterhouse et al., 2005b) time-zone transitions, food intake and its enjoyment were found to be affected more transiently than are the circadian rhythm of core temperature and some other symptoms of "jet lag." The conclusion common to these results is that food intake is comparatively little influenced by the phase of the individual's body clock—that is, food intake has only a weak endogenous component.

Food intake, and responses to it, are instead dominated by exogenous factors, including general lifestyle and culture; within this framework, time pressure (insufficient time to prepare and eat a full meal) and social factors play important roles (Reinberg et al., 1979; Shepherd, 1989; Tepas, 1990; Chiva, 1997; de Castro, 1997; Chapman et al., 1998; Oltersdorf et al., 1999; Popkin et al., 1999; Prattala and Roos, 1999; Winkler et al., 1999; de Castro, 2000 a,b,c; de Castro et al., 2000; Meiselman et al., 2000; Bogdan et al., 2001; Rappoport et al., 2001; Wilson, 2002; Marshall and Bell, 2003; Weber et al., 2004). Previous work by us (Waterhouse et al., 2003a) indicated that the timing, nature, and enjoyment of meals are influenced by work patterns of individuals, not only in those working at night but also in those working during the daytime. These effects are due to the structured nature of the work shift, which can limit the opportunity to eat a cooked meal when hungry. By contrast, individuals tended to eat larger meals, with a higher proportion being hot meals, during rest days when they have a big appetite before meals, enjoyed them more, and were more satisfied afterwards. Consonant with these findings of the difference between "structured" (work) and "unstructured" (rest) days were those of another study which compared a sample of working adults with a sample of older, retired, or semi-retired individuals (Waterhouse et al., 2005a). The

older group differed from the working group on weekdays (when the working group showed effects upon food intake due to time pressure and social constraints); but the two groups did not differ significantly on rest days (when the unstructured days meant that the effects of time pressure and social constraint were absent). There is the general finding, therefore, that meals are larger and enjoyed more in the absence of time pressure and when the meal has a social concomitant. These results have considerable implications for night workers, for example (Reinberg et al., 1979; Tepas, 1990; Waterhouse et al., 1992).

In the current studies, we confirm these results and extend them in 3 ways. First, we have investigated in more detail why the factors of lack of time pressure and a social concomitant to the meal exert positive effects upon subjective responses to meals; is it only because they enable larger meals to be eaten, or do these factors exert effects in addition to their influence upon the meal size? Second, we have considered the difference in meal size between the meal actually eaten and that which would have been chosen in the absence of any constraints; what factors might influence this difference? Third, we have investigated if the results are the same whether "structured" or "unstructured" refers to individual 3 h sections of a single day (and therefore when both structured and unstructured periods might be present in any day) or to the day as a whole (a working *vs.* a resting day, for example).

METHODS

Subjects

Two separate studies were performed on different groups of subjects. The vast majority of the subjects were full-time students at Liverpool John Moores or Birmingham Universities. In the first study, 30 female subjects (age, 20 to 23 yrs) were studied at all times of their menstrual cycle; in the second, 19 females and 7 males (age, 18 to 25 yrs) were studied, the females again being at all stages of their menstrual cycle. Subjects were recruited by two of the authors (F.T., Study 1; L.B., Study 2) and were convenience samples. None was receiving any medical treatment. The subjects were studied during the middle of school term when there was no special pressure of examinations, coursework deadlines, and so on. All subjects had conventional sleeping habits (sleeping at night) and ate meals at or close to conventional times, whenever their schedule permitted this. All procedures wee approved by the Institution's Human Ethics Committee and adhered to the ethical guidelines recommended for the conduct of human biological rhythm research (Touitou et al., 2004). All subjects provided written, informed consent to participate in the investigation.

Investigative Protocol

Subjects in both studies were encouraged to continue their normal routines and to choose 6 days that fulfilled the following criteria:

Study 1: 3 days involving structured activities due to university commitments ("structured days"), and 3 days which did not involve any imposed structure ("unstructured days").

Study 2: each of the days contained some 3 h periods that had structured activities ("structured period") and some that did not ("unstructured period").

Questionnaire

The questionnaire was based on one that has been described previously (Waterhouse et al., 2003a; 2004; 2005a). In summary, its responses provide information about how food intake is affected by habit, social factors, schedule, food availability, motivation, and hunger; it also ascertains information about type of meal chosen and some subjective responses to it. The original questionnaire was modified so that there was an additional question that asked for the type of meal that subjects would have liked to eat in the absence of any constraints.

During the 6 days of each study, subjects answered the questionnaire on food intake at 3 h intervals, covering 06:00 to 09:00 h, 09:00 to 12:00 to h, 12:00 to 15:00 h, 15:00 to 18:00 h, 18:00 to 21:00 h, and 21:00 to 24:00 h in Study 1 and 07:00 to 10:00 h, 10:00 to 13:00 h, 13:00 to 16:00 h, 16:00 to 19:00 h, 19:00 to 22:00 h, and 22:00 to 01:00 h in Study 2. Subjects in Study 1 chose 3 days that they considered to be "structured" (generally weekdays with university work) and 3 considered to be "unstructured" (generally weekends with free time). In Study 2, each 3 h interval of the 6 days was described by the subject as "structured" or "unstructured" (generally for the same reasons as applied in Study 1), each day containing both structured and unstructured components.

The questionnaires were identical on all 36 occasions (6 per day at 3 h intervals, and for 6 days), and consisted of the following sections:

A. Subjects first answered if they had been asleep throughout the previous 3 h, in which case this was the end of the questionnaire for this interval.

B. If they had been awake, they were asked whether or not they had eaten a meal in the last 3 h. If they had not eaten, they were required to state why this was the case (choosing one or more options from "Never do," *"Not hungry"*, *"No time available,"* or "No food available"), and this was the end of the questionnaire for this interval. Had they eaten, then the reasons for doing so (choosing one or more options from "Always do,"

"Hungry", "Bored," or *"Social reasons"*), and the factors determining this choice (chosen from *"Appetite," "Time availability,"* "Cost," or "Food availability") were requested. The italicized options were the ones that have been analyzed in the present report, because they are the most pertinent to the aims of the current investigation. However, the subjects were not told the detailed aims of the investigation, and no options were italicized in the questionnaires.

C. Subjects were then asked to state the kind of meal eaten, choosing from 1 = snack food (crisps), 2 = cold snack (sandwich), 3 = cooked snack (jacket potato), 4 = cold meal (salad), 5 = cooked meal (burger and chips); 6 = 2-course meal (main course and starter or dessert); 7 = 3-course meal (starter, main course, and dessert). Examples of these types of meal were given as a guide, and one of these examples is shown above in the brackets.

D. Subjects were next asked to state the kind of meal they would have liked to eat (meal wanted) if there had been no restrictions (due to lack of time, money, food, and so on), choosing from the same 7 options as in (C).

E. Finally, subjects were asked to estimate their hunger before the meal they ate, their enjoyment of it, and their feeling of satiety afterwards. In each case, a scale from 0 to 10 was used, where: "0" = "not at all hungry," "no enjoyment at all," or "still hungry;" "5" = "moderately hungry," "moderate enjoyment," or "satisfied;" and "10" = "very hungry," "enjoyed very much," or "overfull and bloated," respectively.

The university campus had a range of outlets for food, including self-service machines, local public houses, and cafeterias selling a variety of foods, from snacks to fully cooked meals.

Subjects answered the questions independently of each other and were asked not to refer back to their previous responses. They were not told the detailed purpose of the questionnaire, and were not advised as to the exact meaning to be placed on the options describing the type of meal (see C and D, above). These are important and unresolved issues, and have received considerable debate (Lennernas et al., 1993a, 1993b, 1995; Gatenby, 1997). They are rather less relevant in this study, since the aim was to relate intra-individual differences in food intake to aspects of the waking day, and all that is required was for internal consistency on the part of each subject. Nevertheless, it seems reasonable and uncontroversial to consider that, in passing through the sequence of meals 1 to 7, there were general increases in meal size, in the time required to eat it, and the opportunity it provided for a social function.

Treatment of Results and Statistical Analyses

These are described in more detail in the Results section where appropriate. The Statistical Package for the Social Sciences (SPSS) for Windows, version 11, was used. In brief, the methods included analysis of variance (ANOVA) with 2 fixed factors and Subject as the random variable, or 2-way ANOVA with repeated measures. Corrections for violations of sphericity (Greenhouse-Geisser) and multiple comparisons of differences within a factor (Bonferroni) were used (Atkinson, 2002) where appropriate. For other data, the Friedman's ANOVA, Kolmorogov-Smirnov test, and Wilcoxon's matched-pairs signed ranks tests were chosen (Siegel and Castellan, 1988). Exact p values are given in the text and tables; as a general guide, the α-level of statistical "significance" was set at $p < 0.05$, though values where $0.10 > p > 0.05$ are also reported; $p > 0.10$, evidence not to reject the null hypothesis, is considered not to be statistically significant. p values of "0.000" given by the statistics package are listed as "0.0005" herein (Zar, 1999, p. 83).

RESULTS

The options "food availability" and "cost" were cited very infrequently by subjects as reasons affecting their choice of food. That is, other reasons were important in deciding whether or not to eat and, if deciding to eat, the kind of food selected.

Study 1

Figure 1, top, shows the group means of the fractions of possible occasions when a meal was eaten during the six 3 h intervals on structured and unstructured days. The statistical results are shown in Table 1, top, where the fixed factors were time of day (6 levels) and day type (2 levels). Meals were eaten less frequently in the early morning and evening, and meals were more likely to be eaten during structured than unstructured days. This difference between structured and unstructured days was particularly marked in the morning (and accounts for the significant interaction effect), and was due to "lying-in" rather than eating a meal on unstructured days.

Not all types of meals (options 1 to 7, see Methods) were eaten at all 6 (3 h) intervals during the day, on both types of days, and by all subjects. This restriction was overcome by pooling an individual's results from all times of the day and dividing meal size into "small" (options 1 to 3, see Methods), "medium" (options 4, 5), or "large" (options 6, 7). Two-way ANOVA with repeated measures, with the within-subject factors meal size (3 levels) and day type (2 levels), was used (Figure 2, top, and

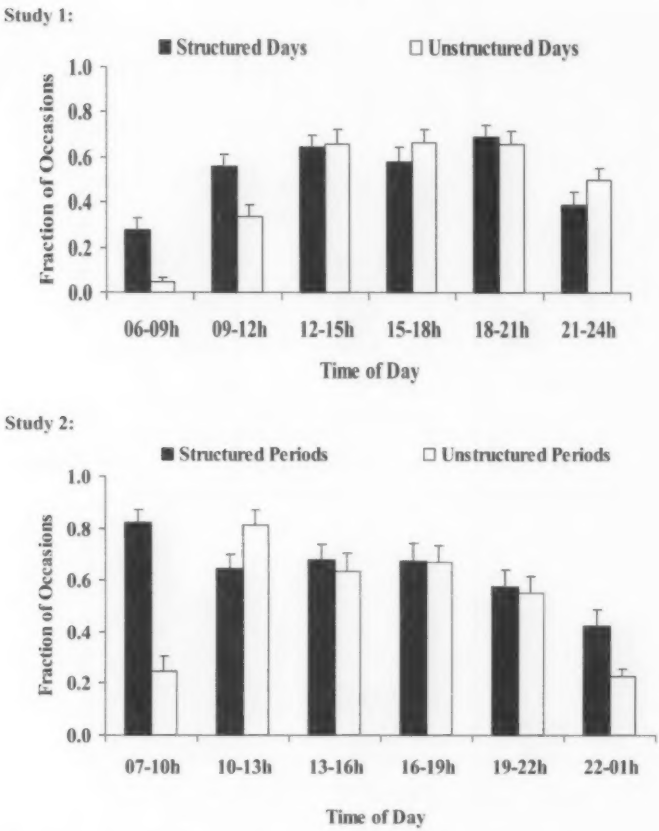

FIGURE 1 Mean (+SE) fractions of possible occasions when subjects ate a meal.

Table 1, top). Results indicate that small meals were eaten most frequently and large ones least frequently. There was an interaction between the frequency of eating meals of different size and structured *vs.* unstructured days; unstructured days were associated with a greater frequency of eating large meals and a smaller frequency of eating small ones.

When the possible reasons for not eating a meal were considered (Figure 3, top), "not hungry" was cited on about half of the possible occasions, and more frequently on unstructured days ($p = 0.004$, Wilcoxon); "no time" was cited about 40% of the possible occasions, but the mean frequencies did not differ significantly between structured and unstructured days. Reasons for eating a meal (Figure 3, top) were dominated by "hungry," this being cited significantly less frequently on unstructured days ($p = 0.002$, Wilcoxon). "Social reasons" was cited less

TABLE 1 Effects of Structured and Unstructured Days or Periods, of Time of Day, and of Size of Meal upon the Frequency of Eating a Meal

Variable	Predictor 1	F-ratio	d.f.	*p*-value	Predictor 2	F-ratio	d.f.	*p*-value interaction	F-ratio	d.f.	*p*-value
Study 1											
F(eat meal)[a]	Day type	**7.05**	**1.0, 29.0**	**0.013**	Time of day	21.33	3.8, 110.3	<0.0005	**3.83**	**3.9,111.8**	**0.006**
F(eat meal)[b]	Day type	1.00	1.0, 29.0	0.33	Size	164.1	1.6, 47.7	<0.0005	*2.67*	*1.6, 45.2*	*0.09*
Study 2											
F(eat meal)[a]	Period type	**7.21**	**1.0, 25.0**	**0.013**	Time of day	11.40	4.1,103.3	<0.0005	**12.28**	**3.4, 85.6**	**<0.0005**
F(eat meal)[b]	Period type	1.41	1.0, 25.0	0.25	Size	71.51	1.8, 45.6	<0.0005	2.25	1.7, 42.0	0.13

Significant results **($P < 0.05$) in bold**; results where *$0.1 > p > 0.05$, in italics*.

[a]Fraction of possible occasions when a meal was eaten.

[b]Fraction of possible occasions when meals of different size were eaten (For more details, see text).

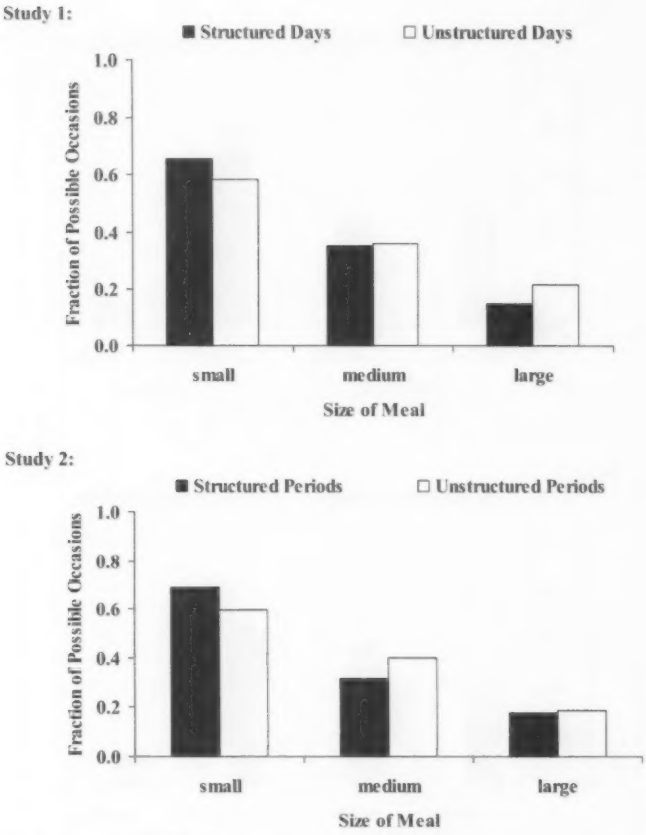

FIGURE 2 Mean fractions of possible occasions that meals of different size were eaten. For definitions of size, see text.

frequently, but the frequency was significantly higher on unstructured days ($p < 0.0005$, Wilcoxon). That is, on unstructured days, the mean likelihood of citing social factors as the reason for eating a meal was increased at the expense of citing hunger.

One of the main aims of the investigation was to establish if some subjective responses to a meal (hunger before the meal, enjoyment of the meal itself, and satiety afterwards) are related to the meal chosen, and if there are separate effects due to day type (structured *vs.* unstructured), social factors, or time availability. These issues were investigated by ANOVA with subjects as a random factor and pairs of fixed factors as possible predictors. (Table, 2 top) As Table 2 shows, size of meals chosen by the subjects was significantly affected by hunger and there was, in

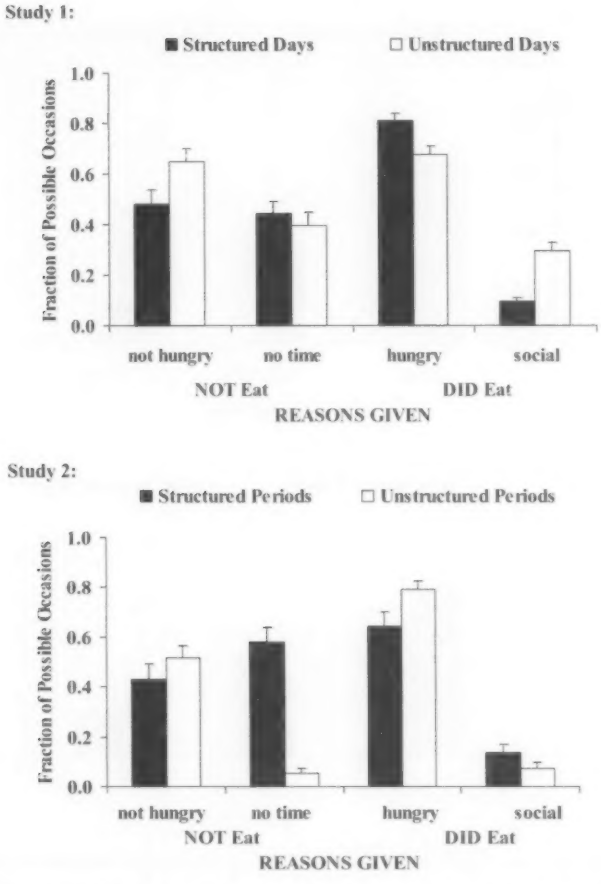

FIGURE 3 Mean (+SE) fractions of possible occasions that reasons for "Not eating" or "Eating" a meal were given.

addition, a significantly reduced meal size for any degree of hunger on structured days (Figure 4, top). Also, for any amount of hunger, the chosen meal size was reduced by the factor "no time" (Figure 5, top) and increased by the factor "social reasons" (Figure 6, top). Table 2, top, also shows that enjoyment of the meals, itself, was directly related to meal size, this effect not differing significantly between structured and unstructured days, and not being separately influenced by time pressure. However, enjoyment was significantly improved when "social reasons" was cited (Figure 7, top), except for the largest meals; this last observation accounts for the significant interaction between social reasons and meal size.

TABLE 2 Factors Influencing the Subjective Responses to Meals

Variable		Predictor 1				Predictor 2			Interaction		
		F-ratio	d.f.	*p* vaule		F-ratio	d.f.	*p* value	F-ratio	d.f.	*P* value
Study 1											
Meal chosen	Hunger	**17.03**	**10.0, 231.0**	**<0.0005**	Day type	**13.04**	**1.0, 45.8**	**0.001**	0.81	7.0, 69.3	0.58
Meal chosen	Hunger	**6.32**	**10.0, 307.4**	**<0.0005**	No time	**6.95**	**1.0, 41.8**	**0.012**	1.01	6.0, 18.2	0.45
Meal chosen	Hunger	**19.25**	**10.0, 325.1**	**<0.0005**	Social reasons	**22.72**	**1.0, 32.7**	**<0.0005**	1.69	7.0, 35.1	0.14
Enjoyment	Meal size	**7.46**	**6.0, 195.4**	**<0.0005**	Day type	0.65	1.0, 94.0	0.42	1.02	6.0, 93.4	0.42
Enjoyment	Meal size	**2.98**	**6.0, 294.1**	**0.008**	No time	1.73	1.0, 42.8	0.20	0.72	3.0, 13.3	0.56
Enjoyment	Meal size	**4.31**	**6.0, 322.6**	**<0.0005**	Social reasons	**8.41**	**1.0, 47.8**	**0.006**	**2.80**	**5.0, 38.9**	**0.030**
Satiety	Meal size	**24.24**	**6.0, 55.4**	**<0.0005**	Day type	2.34	1.0, 104.7	0.13	*1.89*	*6.0, 92.2*	*0.09*
Satiety	Meal size	**7.65**	**6.0, 155.5**	**<0.0005**	No time	**4.87**	**1.0, 37.8**	**0.034**	0.69	3.0, 12.4	0.58
Satiety	Meal size	**14.08**	**6.0, 295.7**	**<0.0005**	Social reasons	2.06	1.0, 38.5	0.16	1.44	5.0, 29.3	0.24
Study 2											
Meal chosen	Hunger	**5.24**	**10.0, 190.7**	**<0.0005**	Period type	1.37	1.0, 34.1	0.25	0.88	8.0, 60.4	0.54
Meal chosen	Hunger	**2.83**	**10.0, 289.2**	**0.002**	No time	**3.36**	**2.0, 49.2**	**0.043**	1.28	9.0, 24.2	0.30
Meal chosen	Hunger		(see text)		Social reasons		(see text)			(see text)	
Enjoyment	Meal size	**4.76**	**6.0, 147.2**	**<0.0005**	Period type	1.31	1.0, 50.9	0.26	1.17	5.0, 67.1	0.34
Enjoyment	Meal size	*1.81*	6.0, 191.6	*0.10*	No time	0.19	2.0, 68.2	0.83	0.80	9.0, 30.5	0.62
Enjoyment	Meal size	3.21	6.0, 132.8	**0.006**	Social reasons	1.29	1.0, 12.8	0.28	*6.70*	*5.0, 2.77*	*0.09*
Satiety	Meal size	**12.14**	**6.0, 157.0**	**<0.0005**	Period type	1.99	1.0, 59.3	0.16	0.78	5.0, 68.2	0.57
Satiety	Meal size	**4.65**	**6.0, 221.8**	**<0.0005**	No time	0.53	2.0, 68.3	0.59	0.23	9.0, 32.4	0.99
Satiety	Meal size	**3.65**	**6.0, 40.1**	**0.006**	Social reasons	0.10	1.0, 12.1	0.75	0.50	5.0, 2.9	0.77

Significant results (**$p < 0.05$), in bold**; results where *$0.10 > p > 0.05$, in italics*.

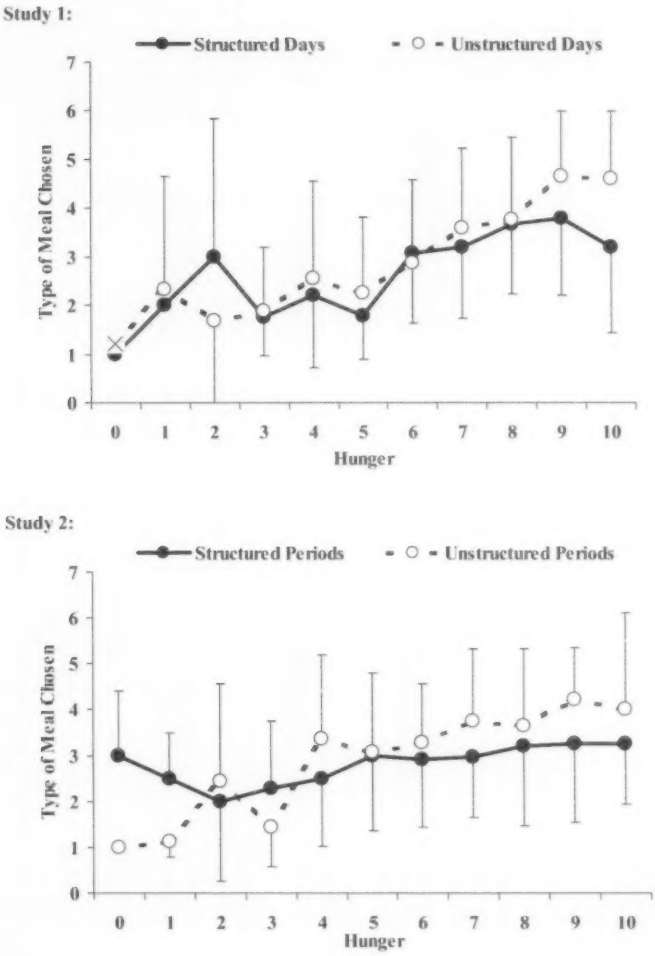

FIGURE 4 Effects of hunger and structured vs. unstructured days/periods upon mean (±SE) type of meal chosen.

Satiety after meals increased with the size of the meal eaten; there was also some evidence for an interaction between meal size and day type, with satisfaction tending to be higher after some meals on unstructured days (Table 2, top). When there was time pressure, satiety was less, but there was no significant effect of social factors. These results all point to the importance of meal size in determining the subjective response to a meal, and indicate that the responses will be affected also by any sense of time pressure or social factors associated with the meal. There is the

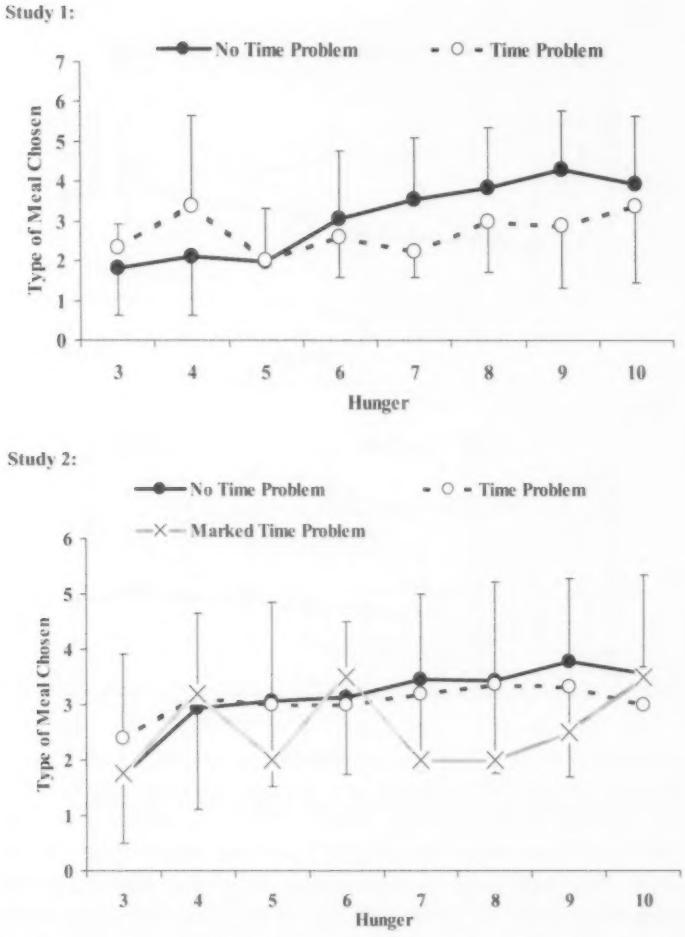

FIGURE 5 Effects of Hunger and Time problems upon mean (±SE) type of meal chosen.

possibility that, in addition, the distribution of meal sizes might vary according to circumstances. These distributions are shown for structured *vs.* unstructured days, absence and presence of time pressure, and absence or presence of social factors in the top section of Figure 8 (left, middle, and right graphs, respectively). The distribution was not significantly affected by day type ($p > 0.10$, Kolmorogov-Smirnov [K-S]). However, there was a significant effect of time pressure ($p < 0.005$, K-S); there was a marked increase in the frequency of eating snacks, at the expense of larger meals. The opposite change took place when the chosen meal had a social connotation ($p < 0.001$, K-S), meal size tending to increase.

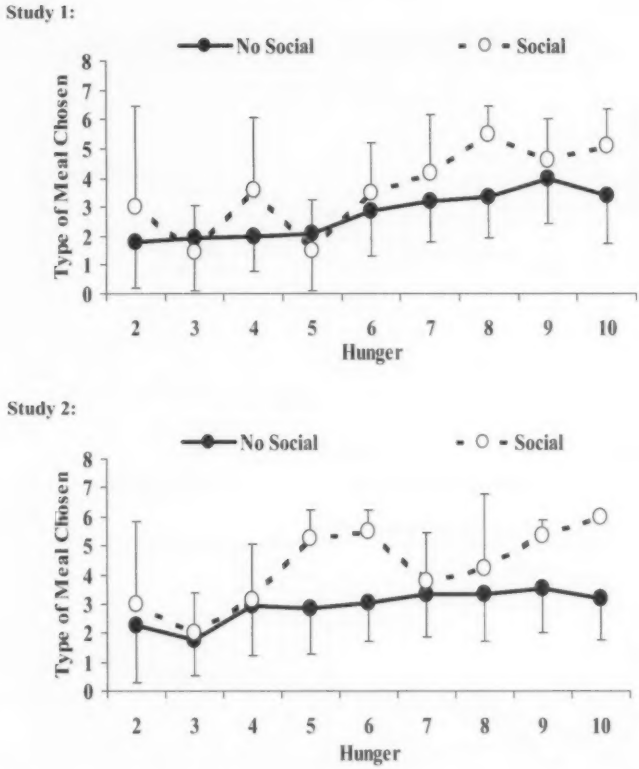

FIGURE 6 Effects of hunger and social factors on mean (±SE) type of meal chosen.

ANOVA also was used to investigate how the individual's hunger affected the meal wanted, in the absence of any time or social constraints, and how the size of this meal affected the sensations of enjoyment and satiety. In these analyses, 'subjects' was a random factor and pairs of fixed factors were possible predictors. The results are summarized in Table 3, top. The size of the meal desired depended upon the hunger before the meal, and was greater on unstructured days. Enjoyment and satiety were dependent upon the size of meal wished for and, again, there was evidence that both variables increased significantly on unstructured days.

Finally, factors affecting the size of the difference between the wanted meal (W) and that actually eaten (E), designated [W-E], were investigated. As predicted, [W-E] was inversely proportional to the size of the meal eaten. However, whereas the 95% confidence intervals for the mean values of [W-E] were significantly greater than zero with smaller meals

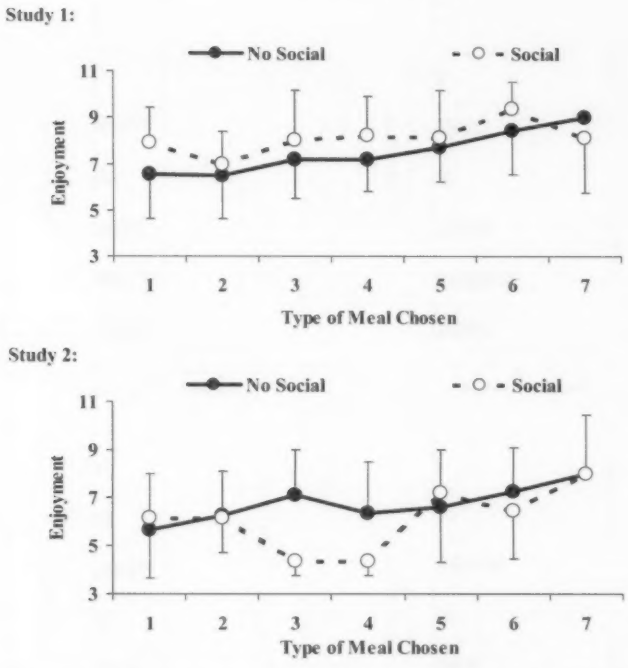

FIGURE 7 Effects of type of meal chosen and social factors upon mean (±SE) enjoyment of meal.

(meal sizes 1 to 4), with larger meals (sizes 5 to 7), these intervals spanned zero. This result indicates that it was thought that meals of sizes 5 to 7 (medium hot meals, 2-course meals, and 3-course meals) would, on average, be of adequate size but that smaller meals would be inadequate. The effect of other factors upon [W-E] is summarized in Table 4. Enjoyment was not significantly affected by [W-E] nor day type, but satiety was inversely proportional to [W-E], even though this result did not differ between structured and unstructured days. [W-E] was not affected by hunger or day type, but it tended to be greater when there was time pressure. When the meals had a social connotation, [W-E] was significantly less; the significant interaction indicates that this reduction in [W-E] importance was more marked on structured rather than unstructured days.

Study 2

In this study, "structured" and "unstructured" refer to 3 h periods rather than to days as a whole (see Methods). The mean frequencies of possible reasons for considering a 3 h period to be structured were

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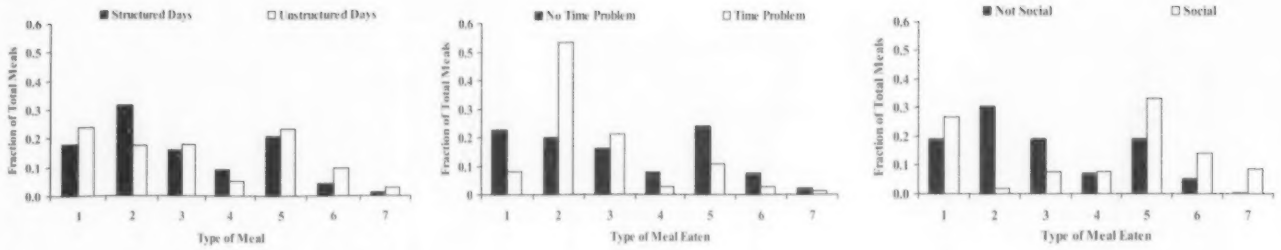

Study 2:

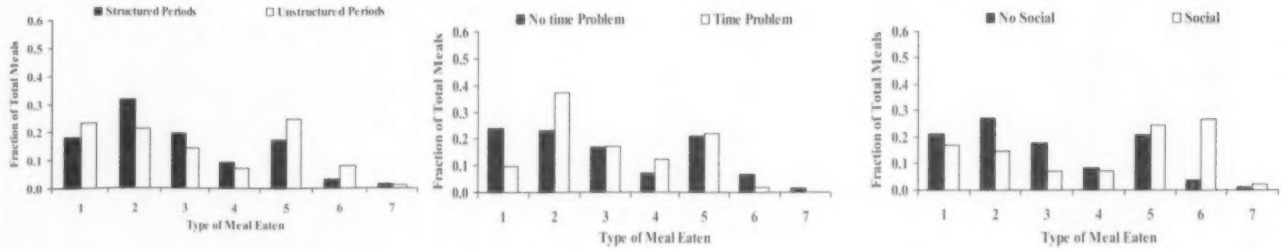

FIGURE 8 Factors influencing the distribution of meals eaten: From left to right: structured *vs.* unstructured days/periods; presence *vs.* absence of time pressure; presence *vs.* absence of social connotations.

TABLE 3 Factors Influencing the Meal Wanted, and the Effect of this Desire upon the Subjective Responses to the Meal Actually Eaten

		Predictor 1				Predictor 2			Interaction		
Variable		F-ratio	d.f.	*p* value		F-ratio	d.f.	*p* value	F-ratio	d.f.	*p* value
Study 1											
Meal wanted	Hunger	**17.83**	**10.0, 229.9**	**<0.0005**	Day type	**7.55**	**1.0, 58.4**	**0.008**	0.37	7.0, 66.5	0.92
Enjoyment	Meal wanted	**7.73**	**6.0, 188.4**	**<0.0005**	Day type	0.09	1.0, 71.9	0.76	*2.08*	*6.0, 100.2*	*0.06*
Satiety	Meal wanted	**14.84**	**6.0, 37.8**	**<0.0005**	Day type	**10.11**	**1.0, 72.8**	**0.002**	1.35	6.0, 91.1	0.24
Study 2											
Meal wanted	Hunger	**8.95**	**10.0, 183.1**	**<0.0005**	Period type	0.28	1.0, 35.0	0.60	1.04	8.0, 59.2	0.42
Enjoyment	Meal wanted	**3.66**	**6.0, 146.7**	**0.002**	Period type	0.03	1.0, 39.8	0.87	0.29	6.0, 43.9	0.94
Satiety	Meal wanted	**4.45**	**6.0, 153.1**	**<0.0005**	Period type	1.84	1.0, 38.6	0.18	0.89	6.0, 51.0	0.51

Significant results **($p < 0.05$) in bold**; results where *$0.10 > p > 0.05$, in italics*.

TABLE 4 Effect of Structured *vs.* Unstructured Days/Periods and Other Factors Upon [W–E][a] and Subjective Responses to a Meal

Variable		Predictor 1				Predictor 2			Interaction		
		F-ratio	d.f.	*p* value		F-ratio	d.f.	*p* value	F-ratio	d.f.	*p* value
Study 1											
[W–E][a]	Hunger	0.94	10.0, 255.7	0.50	Day type	2.10	1.0, 41.2	0.16	0.58	7.0, 68.3	0.77
Enjoyment	[W–E][a]	0.74	7.0, 171.5	0.64	Day type	0.31	1.0, 214.9	0.58	0.17	5.0, 45.7	0.97
Satiety	[W–E][a]	**2.22**	**7.0, 172.4**	**0.035**	Day type	2.51	1.0, 218.5	0.11	0.34	5.0, 50.9	0.88
[W–E][a]	No time	*3.40*	*1.0, 52.6*	*0.07*	Day type	0.26	1.0, 182.1	0.61	2.71	1.0, 8.1	0.14
[W–E][a]	Social	**8.81**	**1.0, 36.8**	**0.005**	Day type	0.08	1.0, 89.5	0.78	**9.00**	**1.0, 18.5**	**0.008**
Study 2											
[W–E][a]	Hunger	2.50	10.0, 192.4	0.009	Period type	1.00	1.0, 43.3	0.32	0.83	8.0, 59.1	0.58
Enjoyment	[W–E][a]	0.68	9.0, 153.6	0.72	Period type	0.14	1.0, 91.4	0.71	1.05	6.0, 46.7	0.41
Satiety	[W – E][a]	0.69	9.0, 159.8	0.72	Period type	0.21	1.0, 104.4	0.65	0.60	6.0, 45.1	0.73
[W–E][a]	No time	1.29	2.0, 58.6	0.28	Period type	0.04	1.0, 82.6	0.84	0.13	2.0, 17.3	0.88
[W–E][a]	Social	0.62	1.0, 17.9	0.44	Period type	1.71	1.0, 58.5	0.20	0.22	1.0, 6.1	0.66

Significant results (**$p < 0.05$) in bold**; results where *$0.10 > p > 0.05$, in italics*.
[W–E][a][Size of meal wanted] minus [Size of meal eaten].

"university business" > "sport" > "social factors" > "paid work" ($p = 0.001$, Friedman's ANOVA). The results are compared below with those from Study 1. When the results were similar, they are reported in less detail than when they were significantly different. The results are detailed in the bottom sections of Figures 1 to 8 and Tables 1 to 4.

The fractions of occasions for eating a meal at different times of the day (Figure 1, Table 1) were distributed similarly to those found in Study 1. The frequencies of eating different types of meals during structured and unstructured periods (Figure 2, Table 1) also were similar to those in Study 1, but the interaction between the meal size and structured *vs.* unstructured periods was not statistically significant.

The reason for not eating a meal, "not hungry," was cited more frequently, but not significantly so ($p = 0.12$, Wilcoxon) during unstructured periods (Figure 3). "No time" was the reason cited most frequently during structured periods, and significantly more so than during unstructured ones ($p < 0.0005$, Wilcoxon). Reasons for eating a meal were mainly "hungry", and this was cited more often during non-structured periods ($p = 0.010$, Wilcoxon). There was no significant difference between structured and unstructured periods when "social factors" were considered ($p = 0.13$, Wilcoxon).

The size of the meal eaten was, as in Study 1, dependent upon hunger before the meal (Figure 4, Table 2), but the difference between structured and unstructured periods was, unlike in Study 1, not significant statistically. There were, again, negative effects of time pressure (Figure 5, Table 2), and Figure 6 indicates that a larger meal was chosen for any degree of hunger when the meal had a social connotation. (The error term could not be calculated by Satterthwaite's method, used in the SPSS package, and so the full analysis was not possible, see Table 2. Even so, pair-wise comparisons indicated that the sizes of meals associated with smaller amounts of hunger were smaller than those associated with larger amounts of hunger, and that the mean meal size in the absence of social influences was significantly, $p < 0.0005$, less than when social factors were present.)

As in Study 1, there was an association between the meal chosen and the enjoyment of the meal, this being uninfluenced by whether the period was structured or unstructured. There was no effect of time pressure, but there was some evidence for a weak effect due to social factors (Figure 7, Table 2).

There was a direct effect of meal size upon satiety after a meal, but no significant effects due to structured *vs.* unstructured periods, time pressure or social considerations (Table 2). These lacks of effect of structured *vs.* unstructured time periods and time pressure were different from those observed in Study 1.

There were differences in the distribution of meals (Figure 8) in the presence *vs.* absence of time pressure (middle graph: $p < 0.05$, K-S) and

social factors (right-hand graph: $p < 0.01$, K-S) that were similar to those found in Study 1. Unlike in Study 1, there was also a trend for more snacks and fewer large meals to be eaten during structured periods (left-hand graph: $p < 0.10$, K-S).

When the meal wanted and the subjective responses to the meal actually eaten were considered (Table 3), there were highly significant effects: of hunger before the meal and the size of meal wanted; of the size of meal wanted and enjoyment of the meal eaten; and of the size of meal wanted and satiety after the meal eaten. These results are the same as those found in Study 1. However, unlike in Study 1, there were no statistically significant effects of structured *vs.* unstructured periods.

Again, the value of [W-E] was inversely proportional to the size of the meal eaten. The 95% confidence intervals for the mean values of [W-E] were significantly greater than zero with meals of sizes (1-3) and (5), and meals of sizes 4 (medium cold meal), 6 (2-course meal,) and 7 (3-course meal) spanned zero. These results are similar to those in the first study. As in Study 1, [W-E] did not depend upon structured *vs.* unstructured periods but, unlike in Study 1, this variable did increase significantly with increasing amounts of hunger (Table 4). Neither structured *vs.* unstructured periods nor [W-E] influenced the sensations of enjoyment or satiety. Satiety was not inversely proportional to [W-E], and there were no significant effects of time pressure or social factors upon [W-E], in which respects the results differed from those of Study 1.

DISCUSSION

Many of the results of Study 1 confirmed our previous studies (Waterhouse et al., 2003a; 2005a). On unstructured (rest) days, for example, there tended to be a "lie-in" (Figure 1, Table 1), and the decision not to eat a meal was often due to time pressure or, particularly on unstructured days, because the individual did not feel hungry (Figure 3). By contrast, the decision to eat a meal was generally because an individual felt hungry; though, on unstructured days social factors became more important (Figure 3). Also, on unstructured days, the type of meal eaten shifted towards eating larger meals rather than smaller snacks (Figure 2). This increase in the size of the meal eaten was associated with an increased hunger before the meal, enjoyment of the meal itself, and satiety afterwards.

The findings of our initial analyses of the data from the studies reported herein indicate that several factors, including the meal size, time pressure, and social opportunities provided by a meal contributed to the greater enjoyment of food on rest days. They confirm the results found in day and night workers (Waterhouse et al., 2003a) and in those who are retired (Waterhouse et al., 2005a), using the same questionnaire.

In a later stage of the analysis, we investigated whether the factors acted simultaneously and independently. These analyses showed not only that meal size was increased when hunger was greater but also that there were additional increases on unstructured, rather than structured, days if there no time pressure or social factors were involved (Figures 2, 4 to 6, Table 2). Furthermore, enjoyment of the meal was increased by larger meals and social factors (Figure 7, Table 2); and satiety after the meal was increased by larger meals and by the absence of time pressure (Table 2). Moreover, the distribution of meals was altered when time pressure was absent or social factors were present, in both cases there being an increased intake of larger meals at the expense of smaller ones (Figure 8).

All these factors (*i.e.*, sufficient time available to eat a meal, being able to choose a larger meal, and the greater possibility of the meal having a social connotation), combine on unstructured (rest) days to make eating a more pleasurable experience. This increased desire for food on unstructured days was supported by the further finding that increased hunger was associated with the individuals stating that the meal they would have wanted to eat (in the absence of any constraints) would have been larger, particularly on an unstructured day (Table 3).

These results concur with those of the literature where a social context has been found to lead to more food being eaten and to the meal being enjoyed more (Shepherd, 1989; de Castro, 1997; Chapman et al., 1998; Oltersdorf et al., 1999; Popkin et al., 1999; de Castro, 2000 a,b,c; de Castro et al., 2000; Meiselman et al., 2000; Rappoport et al., 2001; Wilson, 2002; Marshall and Bell, 2003; Weber et al., 2004). The opposite of these circumstances—eating small meals or snacks, often due to lack of time to eat a full meal, and little social interaction—will, at least partly, account for the altered food intake and lack of enjoyment of meals that are often found in those working night shifts (Reinberg et al., 1979; Tepas, 1990; Waterhouse et al., 1992; Waterhouse et al., 2003a).

Many of the results of Study 2 reinforced those obtained in Study 1. However, there were some differences between the 2 studies. The main difference was that, even though the advantages associated with eating meals on unstructured rather than structured days had been a frequent finding in Study 1, the advantages of unstructured rather than structured periods (lasting for 3 h, see Methods) in Study 2 was less important. This difference between the 2 studies was particularly evident when hunger was considered. To give 3 examples: (1) "not hungry" was often cited in both studies as a reason for not eating a meal, but the frequency of citation in Study 2, unlike the result in Study 1, did not vary between structured and unstructured periods (Figure 3). (2). In both studies, more hunger before a meal increased the meal size eaten, and the meal size was further increased by a lack of time pressure and the presence of social

factors; however, in Study 2 compared to Study 1, there was no significant effect due to structured *vs.* unstructured periods (Figures 4–6, Table 2). (3) The size of the meal wanted in the absence of any constraints was, in both studies, greater as the amount of hunger increased; however, whereas there was a further increase in size on unstructured days in Study 1, meal size did not increase with unstructured periods in Study 2 (Table 3).

One possible explanation of these differences between the 2 studies is as follows. In Study 1, defining a day as "unstructured" did not mean that no part of it was structured or that no tasks had to be carried out; it meant instead that, considered as a whole, there would have been sufficient time and opportunity to plan a meal (which was likely to have been substantial in size and content) and to have invited friends to share in its enjoyment. Also, considering a day to be "structured" did not mean that there was no free time, but it did imply that there would be insufficient time during the course of that day to make all the preparations that would have been necessary for a substantial meal. In other words, using the whole day as a frame of reference gives the individual some idea of the opportunities that will exist during that day for meeting friends and enjoying a substantial meal. Experience and culture presumably would lead an individual to associate such opportunities with rest rather than work days (Shepherd, 1989; Chiva, 1997; de Castro, 1997; Chapman et al., 1998; Oltersdorf et al., 1999; Popkin et al., 1999; Prattala and Roos, 1999; Winkler et al., 1999; de Castro, 2000a,b,c; de Castro et al., 2000; Meiselman et al., 2000; Bogdan et al., 2001; Rappoport et al., 2001; Wilson, 2002; Marshall and Bell, 2003; Weber et al., 2004). By contrast, if "structured" and "unstructured" refer to the last 3 h interval only, then both types of period might be part of an otherwise unstructured (rest) or structured (work) day; in this context, food intake during a particular 3 h period could not be related to an individual's general expectations and plans with regard to the day taken as a whole. As the current results show, individuals were more likely not to eat a meal when they were under time pressure and, if they did eat a meal, then it was less likely to be due to hunger and probably more likely to be because it fitted in with their schedule (Figure 3). These factors could apply during both the structured and unstructured periods of the day, and so could contribute to the lack of differences between such periods that was found in Study 2. Nevertheless, the size of meal eaten could still depend upon the amount of hunger and enjoyment of the meal, and the satiety after it could still be related to the meal size, and social factors could still be important—as was found in the current investigation (Figures 4, 6, 7, Table 2). In other words, when meals and subjective responses to them are considered, differences between structured and unstructured 3 h periods are likely to be far less important to individuals than are differences between whole days.

In conclusion, several factors combine to influence food intake, including time pressure, social considerations, and whether it is a structured or unstructured day. These factors also affect subjective responses to food, such as hunger before a meal, enjoyment of the meal itself, and satiety afterwards. In practice, the positive aspects of these factors can act together on rest (unstructured) days; a substantial meal can be prepared, and it can be appreciated more in the company of friends and without the problems of time pressure. The converse of this (*i.e.*, that the differences between responses to meals might be reduced if a time-scale less than a whole day is considered) might be applicable to night workers; for example, it might be of value for them to consider that a meal during the night shift is associated with only part of the day (their period of work), rather than with a whole day that is abnormally phased with regard to their families and the rest of society.

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Chronobiology International, 22(6): 1093–1106, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398064

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MELATONIN ENTRAINS FREE-RUNNING BLIND PEOPLE ACCORDING TO A PHYSIOLOGICAL DOSE-RESPONSE CURVE

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The specific circadian role proposed for endogenous melatonin production was based on a study of sighted people who took low pharmacological doses (500 μg) of this chemical signal for the "biological night": the magnitude and direction of the induced phase shifts were dependent on what time of day exogenous melatonin was administered and were described by a phase-response curve that turned out to be the opposite of that for light. We now report that lower (physiological) doses of up to 300 μg can entrain (synchronize) free-running circadian rhythms of 10 totally blind subjects that would otherwise drift later each day. The resulting log-linear dose-response curve in the physiological range adds support for a circadian function of endogenous melatonin in humans. Efficacy of exogenous doses in the physiological range are of clinical significance for totally blind people who will need to take melatonin daily over their entire lifetimes in order to remain entrained to the 24 h day. Left untreated, their free-running endocrine, metabolic, behavioral, and sleep/wake cycles can be almost as burdensome as not having vision.

Keywords Melatonin, Blindness, Free-running circadian rhythms, Endogenous circadian period (tau), Melatonin entrainment dose-response curve

INTRODUCTION

In recent years, there has been considerable interest in the molecular mechanisms of the biological timing system, including the clock genes and transcriptional feedback loops that are the basis of circadian rhythms, as well as the novel retinal photoreceptors required for entraining (synchronizing) the master hypothalamic pacemaker to the 24 h day

Submitted September 17, 2005, Returned for revision September 21, 2005, Accepted September 28, 2005

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(Panda et al., 2002; Reppert and Weaver, 2002; Hattar et al., 2003; Menaker, 2003). These photoreceptors also mediate light suppression of pineal melatonin production, ensuring that high levels are confined to nighttime darkness (Lucas et al., 1999). Stimulation of melatonin receptors (Gerdin et al., 2004) in hypothalamic nuclei neurochemically converts the light/dark cycle into seasonal and circadian signals (Axelrod, 1974; Moore and Klein, 1974).

In humans, active pineal melatonin production occurs for about 12 continuous h out of every 24 h. The beginning of each circadian cycle is termed the melatonin onset (MO), and it is a reliable phase marker for the entire endogenous melatonin profile, as well as for all of the circadian rhythms regulated by the hypothalamic pacemaker located in the suprachiasmatic nucleus (SCN) (Axelrod, 1974; Moore and Klein, 1974; Lewy et al., 1999). Melatonin production is an ideal marker for circadian phase, because it is relatively free from perturbations. The major one in sighted people is light exposure, which suppresses melatonin levels: the brighter the light, the greater the suppression (Lewy et al., 1980). Sufficiently bright light exposure during the night, as well as at the beginning and end of the diurnal activity span, can suppress melatonin production; however, a bout of darkness during the day does not stimulate melatonin synthesis (in both sighted and blind people, the SCN inhibits its production for about 12 h during each circadian cycle). In sighted people, samples need to be collected under dim light (<30 lux) in order to use the melatonin profile as a marker for the phase of the SCN; accordingly, the dim light melatonin onset (DLMO) is most often used in human chronobiologic studies (Leibenluft et al., 1996; Lewy et al., 1997; Voultsios et al., 1997; Lewy et al., 1998; Nagtegaal et al., 1998; Gordijn et al., 1999; Lewy et al., 1999; Lewy et al., 2001; Lewy et al., 2002; Lewy et al., 2003; Emens et al., 2005).

Although humans lack annual reproductive cycles, they have retained the light suppression (Lewy et al., 1980) important for what we have proposed to be the physiological role of melatonin—augmenting entrainment of the endogenous circadian pacemaker by the light/dark cycle to the 24 h day (Lewy et al., 1992). This hypothesis was based primarily on time-of-day-dependent phase shifts to 500 μg of melatonin in diurnally active sighted people: according to its phase-response curve (PRC), melatonin causes phase delays (shifts to a later time) when administered in the morning and causes phase advances (shifts to a earlier time) when administered in the afternoon/evening (Lewy et al., 1992). The advance and delay zones are about 12 h in duration and about 12 h out of phase with those of the light PRC: for both PRCs, the cross-over points between these zones are the same times—although reversed (Lewy et al., 1998). The melatonin PRC described in sighted people appears to be generalizable to those lacking light perception (Sack et al., 2000; Lewy et al., 2001; Lewy et al., 2002; Lewy et al., 2003).

About 15% of the legally blind are unable to detect light. Although many are able to adhere to conventional sleep schedules, their endogenous circadian rhythms typically free-run with circadian periods (taus or τs) that are almost always greater than 24 h. When free-running, the melatonin onset (MO)—the daily rise in circulating melatonin levels—occurs later in the day with each successive circadian cycle. Measured either in plasma or saliva, the MO indicates the phase of the hypothalamic endogenous pacemaker and its driven circadian rhythms. As mentioned above, BFRs also have approximately12 h intervals of active and quiescent production. When the MO is not in normal phase ($\sim$ 20:00 h in sighted people), the resulting sleep, alertness, and mood problems are a great burden to many BFRs—second only to the absence of vision (Sack et al., 2000). Daily administration of exogenous melatonin can successfully entrain them (Sack et al., 2000; Lewy et al., 2001; Hack et al., 2003), similar to what has been reported in comparable animal studies (Redman et al., 1983).

We (Sack et al., 2000; Lewy et al., 2001; Lewy et al., 2002; Lewy et al., 2004) and others (Hack et al., 2003) have shown that 0.5 mg (500 μg) can entrain BFRs. In fact, this dose was able to provide the requisite 0.9 h daily phase advance to entrain a BFR with a long (24.9 h) τ, who failed to entrain on higher doses of 10 and 20 mg (discussed in further detail later). We have previously reported successful entrainment of BFRs to 50 μg stepped down to 25 μg (Lewy, 2003; Lewy et al., 2004). The following is the first report of a group of BFRs entrained to doses of melatonin in the physiological range. Two hypotheses were tested: 1) physiological doses of melatonin cause daily phase shifts according to a log-linear dose-response curve; and 2) BFRs with longer τs require higher physiological doses to entrain. Some of these subjects had previously participated in other melatonin-treatment studies; for example some were previously treated with 10 mg (Sack et al., 2000).

SUBJECTS AND METHODS

Subjects

Ten totally blind adults (Table 1) were recruited from blind advocacy organizations or by referral within the blind community. Information about the study was provided to subjects in print, Braille, and/or audiotape; they gave written informed consent. The Oregon Health & Science University (OHSU) Institutional Review Board approved the protocol and consent forms, and its design and conduct adhered to the ethical guidelines of the journal outlined for human biological rhythm research (Touitou et al., 2004). All subjects were in good general health, had no conscious perception of light, and were not taking any medications that

TABLE 1 Subject Demographics and Melatonin Data

Subject (no.)	Age (Y) (yrs)	Sex	Lowest entraining dose (μg)	Time of melatonin administration[a]	Time of entrained MO[a]	τ at entrainment phase (TEP)[b]	Linear regression τ[b]	Entrained τ[b]
1	50	F	20	19:30	20:40	24.16	24.20 ± 0.13	23.994 ± 0.02
2	35	M	50	19:00	23:56	24.47	24.54 ± 0.05	24.009 ± 0.026
3	58	M	50	17:00	18:03	24.32	24.34 ± 0.07	23.994 ± 0.076
4	48	F	75	18:00	21:11	24.19	24.30 ± 0.04	24.001 ± 0.002
5	53	M	75	19:00	19:34	24.34	24.33 ± 0.01	23.998 ± 0.005
6	54	F	75	18:30–20:00	22:43	24.65	24.54 ± 0.10	23.997 ± 0.004
7	50	M	100	18:00	20:20	24.32	24.34 ± 0.02	24.013 ± 0.016
8	54	F	200	19:00	22:03	24.59	24.57 ± 0.02	23.998 ± 0.005
9	70	F	200	18:00	20:10	24.40	24.43 ± 0.06	23.990 ± 0.025
10	49	M	300	17:00	19:01	24.89	24.89 ± 0.16	24.002 ± 0.004

[a]Clock hour (shown as hours:minutes).
[b]Hours.
MO = Melatonin onset.

would compromise accurate determination of the endogenous MO, with the exception of Subject 2, who was taking a beta-blocker.

Measurement of Endogenous Pineal Melatonin Production

During each one-day admission to the OHSU General Clinical Research Center (GCRC), blood or saliva was obtained at 1 h intervals for at least 24 h, although in some instances when entrainment had been established, the sampling window was limited to 6–12 h. We did not specifically restrict activity or posture, although most subjects were recumbent during sample collections. The finding of previous studies (Voultsios et al., 1997; Cajochen et al., 2003) indicates posture does not appear to affect the MO. Blood was drawn through an intravenous saline-lock, usually placed in the forearm. Following centrifugation (1400–1500 g), plasma was decanted into fresh tubes and frozen at −20°C. Saliva was collected using Salivettes® (SARSTEDT, Inc., Newton, NC, USA), which involved chewing (chewing stimulates salivation) on a dental absorbent cotton plug; after about 30–60 sec, it was fully saturated The plug was then transferred directly from the mouth to an inner plastic centrifuge tube, with a hole at its bottom, contained in a larger capped plastic tub. A clear filtrate at the bottom of the larger tube, resulting from centrifugation at 1400–1500 g, was then frozen and stored at −20°C for a few days until assayed for melatonin. Plasma and saliva samples were thawed for assay usually within a few days of collection. Melatonin concentrations were measured by radioimmunoassay using an antibody raised in the laboratory of Kennaway and co-workers (Earl et al., 1985; Voultsios et al., 1997) and reagents supplied by ALPCO Diagnostics (Windham, NH, USA). This assay has a lower sensitivity limit of 0.5 pg/ml, and it was validated by GCMS (Lewy and Markey, 1978; Lewy et al., 1997).

Determination of MO

Around the time of the MO, melatonin levels in saliva are about one-third those of plasma. Therefore, a 0.7 pg/ml salivary level is equivalent to 2 pg/ml plasma concentration (Voultsios et al., 1997; Emens et al., 2005). By operationally defining the MO as the interpolated time when melatonin levels continuously rose above these low thresholds, the potential confound of inter-individual differences in the amplitude of melatonin production was avoided (Lewy et al., 1999). In one subject (No. 8) whose endogenous melatonin amplitude decreased dramatically during the course of the study, a lower threshold (plasma 0.7 pg/ml) was used for MOs during entrainment. This threshold was comparable to the plasma 2 pg/ml threshold used in her free-running data.

The plasma 2 pg/ml (salivary 0.7 pg/ml) MO occurs on average at ~20:00 h in sighted people (Lewy, 2003).

Calculation of Circadian Period (τ) from Sequential MOs

The baseline τ was calculated by fitting a least-squares linear regression to a minimum of 4 MOs assessed every few weeks through at least one complete beat cycle, i.e., when the MO had traversed across 24 h. As shown in Table 1, the shortest baseline (free-running) τ was 24.20 ± 0.13 h [$\tau \pm$ 95% Confidence Interval (CI)] and the longest was 24.89 ± 0.16 h. During exogenous melatonin treatment, τ was calculated from at least 4 consecutively determined MOs. Criteria for entrainment required that $\tau \geq 23.96$ and ≤ 24.04. In accordance with our prior studies (Lewy et al., 2004), it was also required that the 95% CI overlap 24.00 h, thus making τ statiscally indistinguishable from 24 h. Resumption of a $\tau \geq 24.04$ indicated escape from entrainment.

Tau (τ) at Entrainment Phase (TEP)

As mentioned above, the average baseline τ was calculated by fitting a linear regression to the MOs of at least 1 beat cycle. However, each two-point τ (the slope of the line connecting two consecutive MOs, plus 24 h) was usually different from the average τ. Two-point τs varied (to a greater or lesser extent in each BFR) in accordance with a predictable, replicable, and generalizable pattern (relative coordination) as the MO traversed across the clock times of the 24 h day (Emens et al., 2005). Tau is usually shorter than the BFR's average when the MO occurs at night, and it usually longer than average when the MO occurs during the day (Emens et al., 2005). This variation could equally well, or perhaps even better, be described as the effect of unknown weak zeitgebers that induce net daily phase-advance shifts and/or net daily phase-delay shifts. The plotting of each two-point τ observed during treatment-free intervals against the average clock time of the corresponding pair of MOs results in a τ-response curve for each BFR. The cross-over times of these τ-response curves are generally near 08:00 and 20:00 h, though the exact times vary among BFRs. The greatest variability, however, is in the amplitude of these curves; that is, some BFRs. appear to be more sensitive to weak zeitgebers than others (Emens et al., 2005).

Each BFR's τ-response curve and the clock time of the MO at entrainment were used to calculate τ at entrainment phase (TEP). TEP is not τ at entrainment, which is, by definition, 24.0 h; rather, TEP is the τ that was observed when the free-running MO occurred at the same clock time at which entrainment subsequently took place. Therefore, subtracting 24 h from TEP provides the most accurate estimate of the magnitude of the

daily phase advance resulting exclusively from the lowest melatonin dose required for entrainment.

Daily Administration of Exogenous Melatonin

Gelatin capsules, identical in appearance and taste, contained lactose filler with or without melatonin and were ingested daily, except on the day of endogenous MO assessment. Subjects did not become sleepy after taking the low doses used in this study, ensuring that they could not distinguish between treatments. GMP grade melatonin (administered under Investigational New Drug Application 26,318) was obtained from Regis Chemical Co. (Morton Grove, IL, USA), and doses were formulated under the supervision of a research pharmacist. The capsule containers were coded and labeled individually for each day of treatment for the subjects in both print and Braille; later in the study, this system was replaced by the use of MEMS-6 TrackCaps® (Aprex Corp., Union City, CA, USA). To assess compliance, capsule bottles were returned at each GCRC admission; any remaining capsules were noted and new capsule bottles were issued. Subjects were told that if they did not remember to take a capsule at the designated time, they could take it up to 2 h later, but to otherwise refrain from taking it that day. Subjects also were instructed to report any missed or late capsules.

The pharmacokinetics of the immediate-release formulation used in this study at a dose of 0.5 mg have been previously reported (Lewy et al., 1998). Formulated in lactose-filled capsules, doses of 20–300 μg can be expected to produce peak plasma melatonin levels ~15–60 min after oral ingestion, usually no greater than ~40–600 pg/ml, respectively (Lewy et al., 1998). There is relatively little intra-individual variability in the amplitude and duration of endogenous melatonin profiles; however, amplitude can differ between individuals across a range of 2–3 orders of magnitude (Lewy et al., 1999). Although peak plasma melatonin levels are usually between 25 and 80 pg/ml (Lewy et al., 1999), they are often less than 20 pg/ml (sometimes as low as 1 pg/ml) and occasionally exceed 100–200 pg/ml. Given the close anatomical proximity between the pineal gland and the hypothalamus, as well as the CSF circulation between them, hypothalamic melatonin receptors may actually be exposed to endogenous melatonin levels that are perhaps 20 times greater than that measured in plasma samples (Skinner and Malpaux, 1999). Also, even the highest of the lowest entraining doses in the present study can be expected to elevate melatonin levels for a much shorter duration than the typical 12 h resulting from endogenous production (Lewy et al., 1998; Lewy et al., 1999; Sack et al., 2000). Hence, it is unlikely that any of the doses were supraphysiological.

Treatment Protocol

An investigator (AJL) not in contact with the subjects reviewed the MO plots updated within a few days of each GCRC admission and decided whether to initiate, continue, or change a melatonin dose, based on the available data and the criteria for entrainment described above. The initial dose for each subject was selected from a series of doses (500, 400, 300, 200, 100, 75, 50, 25, and 20 μg), and the goal was to eventually entrain the subject at a normal circadian phase using the lowest effective melatonin dose. For each subject, this was achieved through a succession of dose changes that involved either a step down to lower dose in the series described above (when entrainment to a given dose was demonstrated) or a step up to a higher dose (when entrainment to a given dose had failed). Pill times were adjusted as needed to ensure that the melatonin onset was occurring at a normal time, relative to the subject's preferred sleep schedule.

Eight out of 10 subjects were initially entrained with pill administration times at late clock (hours between 21:00 and 01:00 h); pill times were then advanced to earlier administration times (between 17:00 and 20:00 h), and doses were stepped down until entrainment was lost or in the case of subject No. 1, the lowest dose reached. Doses of subjects who escaped entrainment were stepped up until the subjects re-entrained with the earlier pill time of administration. Subject 1 maintained entrainment when stepped down to the lowest dose in the series (20 μg), and it is therefore possible that her lowest entraining dose could be lower. Subject 3 and 7 did not entrain the initial dose of 25 μg at early pill times (17:00 and 18:00 h, respectively), and were stepped up through the series (until entrainment occurred on 50 μg and 100 μg, respectively).

Choice of Melatonin Administration Clock Times

In order to reduce the influence of inter-individual differences in relative coordination, we selected final melatonin administration times between 17:00 and 20:00 h, thus ensuring that the entrainment phase of the endogenous MO was reasonably close to 20:00 h (the range in the 10 subjects was 18:03–23:56 h). When the MO occurs at these clock times, inter-individual differences in the effects of weak zeitgebers are optimally taken into account—although the use of TEP (rather than average τ) adequately addresses this potential confound. In any event, these administration times helped achieve the first two of the three therapeutic goals, i.e. 1) entrainment 2) at the normal phase 3) using the lowest effective dose.

In order to ensure the understanding of the study methods, the MO data for the last part of the protocol appears in Figure 1 for Subject 8. The first part of the protocol (baseline τ assessment and entrainment to

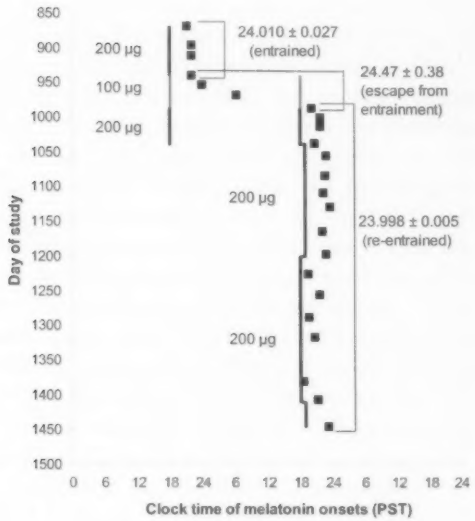

FIGURE 1 Determining the lowest dose capable of entraining a BFR. Day of study participation for Subject 8 is plotted on the ordinate [the first part of the protocol (baseline τ assessment and entrainment to doses of 500, 400, and 300 µg) is not shown, which is why the study days begin at 850]. On the abscissa is Pacific Standard Time, which repeats every 24 h in order to display the gradual drift of active melatonin production to later and later times as marked by the time of the melatonin onset, MO (■). Each vertical bar indicates the time and duration of melatonin administration, and the dose appears on the left. The vertical bar is repeated for days 941-1039. Pacific Daylight Time transitions are indicated by an apparent 1 h shift in administration time, which was actually held constant at 19:00 h. Circadian periods (τs) ± 95% CIs are indicated for each bracketed condition. The average baseline (pre-treatment) τ was 24.57 ± 0.02 h.

doses of 500, 400, and 300 µg) is not shown, which is why the study days begin at 869.

RESULTS AND DISCUSSION

We were able to find a lowest entraining dose of melatonin for each of the 10 subjects, and each was in the physiological range of 20–300 µg. The plot in Figure 2 is the first melatonin dose-response curve described in BFRs. The ordinate indicates the daily phase advance required for entrainment of each BFR. These values were calculated by subtracting 24 h from the TEP of each BFR. Therefore, they indicate the phase advance caused by each dose that appears on the abscissa: these doses are the lowest capable of entraining each BFR.

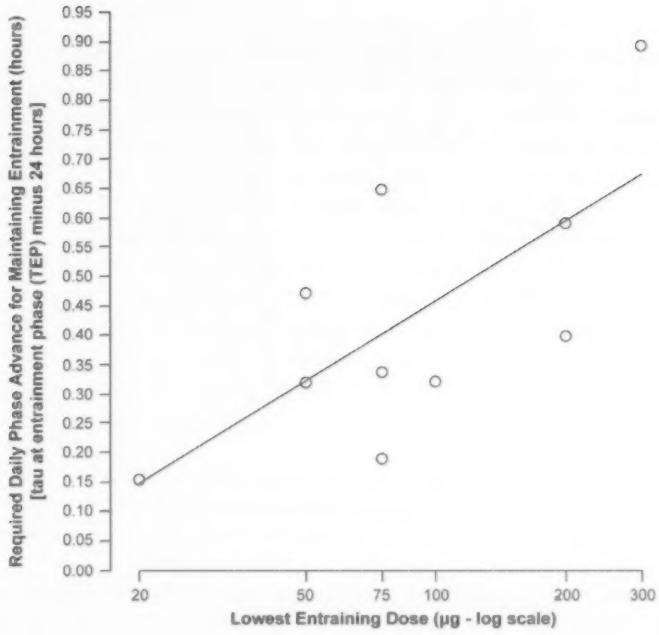

FIGURE 2 Log-linear relationship between the lowest entraining dose and the daily phase advance of 10 BFRs. The daily phase advance was calculated by subtracting 24 h from the TEP (see text) assessed during the pre-treatment free-run. A regression line was fit to the data, and a Pearson's correlation was found to be statistically significant ($r(8) = 0.69$, $p = 0.026$). The τ for each BFR can be calculated by adding 24 h to the ordinal data values, and the linear regression, comparable to the one above, supports our other hypothesis that greater daily melatonin doses are required to entrain BFRs with longer τs. Doses in the range of 20–300 µg produce plasma melatonin levels that are similar to those produced by the pineal gland, which provides support for the proposition that endogenous melatonin has a circadian function in humans.

A Pearson correlation indicated a relationship between the required daily phase advance and the log of the lowest entraining dose that was statistically significant ($T(8) = 0.69$, $p = 0.026$). The Kendall rank correlation was also significant ($\tau(8) = 0.52$, $p = 0.044$), but the Spearman was not ($r_s(8) = 0.62$, $p = 0.054$). The use of average τ, instead of TEP, for calculating the daily phase shifts on the ordinate also resulted in a significant Pearson correlation ($r(8) = 0.71$, $p = 0.021$); however, neither the Kendall nor the Spearman rank-order tests were significant ($\tau(8) = 0.47$, $p = 0.067$; $r_s(8) = 0.57$, $p = 0.083$). Therefore, it may be important to take into account the effect of weak zeitgebers on tau when assessing the phase-shifting effects of exogenous melatonin as it entrains the MO to a particular time of the day.

In a study of sighted individuals (Deacon and Arendt, 1995), only the lowest of their one-day doses (0.05, 0.5, and 5.0 mg) of melatonin that caused phase advances was within the physiological range; however, although not statistically meaningful, the average phase-advance values for all 3 doses appear to be consistent with our finding, when included with our data in the log-linear plot in Figure 2. The experimental paradigm of the present study, with its unambiguous endpoint of success/failure for entrainment, may be the only way to precisely measure the phase-resetting effects of the very lowest doses of melatonin. We speculate that the correlations reported above would improve with the use of more than 6 doses in the 20–300 μg range.

In all of our studies to date, BFRs who entrained to melatonin required no more than 300 μg. In the first study that included Subject 10, the 10 mg dose failed to entrain him (although it was able to shorten his τ from 24.9 to 24.3 h); however, it entrained the other 6 BFRs whose τs ranged from 24.2 to 24.6 h, suggesting that this dose could cause a maximal daily phase advance of 0.6 h (Sack et al., 2000). In a subsequent study (Lewy et al., 2002), we showed Subject 10 could not be entrained to a dose of 20 mg, whereas, a dose of 0.5 mg (500 μg) caused the 0.9 h phase advance required for entrainment. We have proposed an explanation for this finding based on the melatonin PRC: higher doses administered on the advance zone are more likely than lower doses to result in elevated melatonin levels that persist for a long span of time before their half-life decay and thus spill over onto the delay zone, thereby reducing the otherwise greater expected phase advance from the higher dose. In the first study that included Subject 10, 3 of the other BFRs (with τs of 24.2, 24.3, and 24.4 h) underwent a step wise dose reduction to 0.5 mg after entrainment to 10 mg without loss of entrainment (Sack et al., 2000). This finding suggested that less melatonin might be needed for maintaining than for initiating entrainment. However, in a follow-up study (Lewy et al., 2001), we successfully captured the free-running rhythms of these BFRs with a *de novo* dose of 0.5 mg; in this and other reports (Sack et al., 2000; Lewy et al., 2002), we suggested that BFRs with longer τ might require greater doses for entrainment, which could be inferred—but to the best of our knowledge has never been shown—from animal studies (Pittendrigh and Daan, 1976).

The present study supports this hypothesis, albeit at a lower dose range than we first envisioned: when 24 h is added to the ordinal data values in Figure 2, the resulting plot indicates that the longer the τ, the greater the melatonin dose required for entrainment. Given that the TEPs (and average pre-treatment τs) encompass a range of almost 1 h and given their importance in calculating meaningful estimates of the daily phase advance required for entrainment, it seems unlikely that entrainment causes significant lingering changes in τ, sometimes called

aftereffects (Moore-Ede et al., 1982). Furthermore, there is no evidence in the present study to support the idea that more exogenous melatonin is required for initially capturing free-running rhythms than is required for maintaining entrainment.

The closer a BFR's pre-treatment τ is to 24 h, the worse may be their circadian burden (Lewy et al., 2001). If $\tau = 25$ h, the circadian sleep propensity rhythm will be 12 h out of phase with desired sleep times every 24 days, but recurrent symptoms will last only for a few weeks. However, if $\tau = 24.1$ h, it takes 240 days to complete a beat cycle, and recurrent symptoms could persist for several months. In either case, melatonin must be taken daily over an entire lifetime. Since conclusive evidence for the long-term safety of daily melatonin may require decades, it is critical that BFRs take the lowest effective dose. Furthermore, low-dose melatonin can be administered in the early evening without inducing sleepiness—melatonin's most significant known side effect, particularly at doses greater than those used in this study (Zhdanova et al., 2001). While sighted people can use bright light for treating their circadian phase disorders, the efficacy of low-dose melatonin should be of benefit to them as well. However, the efficacy of physiological doses is of particular importance in the treatment of totally blind infants and children, in whom endocrine, metabolic, behavioral, and sleep/wake circadian rhythms entrained at normal phase would be optimal for growth and development.

Effective phase-resetting with physiological doses suggests a role for endogenous melatonin in the human circadian system (Lewy et al., 1992), consistent with animal studies (Cassone et al., 1986; Warren et al., 1993; Cassone, 1998). Our hypothesized function for endogenous melatonin in humans (Lewy et al., 1992) is similar to what Gwinner and Brandstatter (2001) have suggested in some avian species. Melatonin as an "internal zeitgeber," proposed by Armstrong (1989) is also supported.

ACKNOWLEDGMENTS

We thank the research subjects, the nursing staff of the OHSU GCRC, Rick Boney, Nancy Stahl, Rebecca Bernert and Brant Hasler, as well as Drs. Robert Sack, Kyle Johnson, Paul Giger and Mark Kinzie. Many thanks to Keith Parrot, Pharm.D., for preparation of melatonin capsules.

This work was supported by grants from the Public Health Service (R01 MH56874, R01 AG21826 and R01 HD42125 to Dr. Lewy; K23 RR17636 to Dr. Emens; and 5 M01 RR000334 to the General Clinical Research Center of OHSU) and the National Alliance for Research on Schizophrenia and Depression (2000 NARSAD Distinguished Investigator Award to Dr. Lewy).

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Chronobiology International, 22(6): 1107–1119, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398080

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SEASONAL VARIATION IN AMOUNT OF UNABSORBED DIETARY CARBOHYDRATE FROM THE INTESTINE AFTER BREAKFAST IN JAPANESE SUBJECTS

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We previously showed that daytime dim-light exposure has a negative effect on the efficiency of dietary carbohydrate absorption in the evening, whereas evening-time dim-light exposure has a beneficial effect. These results suggest that seasonal changes in the environmental light may affect gastrointestinal activity, and that there might, therefore, be seasonal variation in the efficiency of dietary carbohydrate absorption from the intestine. In order to prove this hypothesis, we measured the amount of dietary carbohydrate unabsorbed from the intestine after a breakfast in healthy female Japanese subjects during the four seasons of the year. We estimated the amount of unabsorbed dietary carbohydrate by the breath hydrogen test, which measures the amount of hydrogen in exhaled air. A 6 g solution of lactosucrose, an indigestible trisaccharide, was used for comparison. Two groups of subjects, 12 subjects in Osaka and 14 subjects in Nagano, were studied in the winter (January to February), spring (April to May), summer (July to August), and autumn (October to November) of 2004. We found the following results: (1) In no season were there any significant differences between the two subgroups in the orocecal transit time of the breakfast and the lactosucrose solution. Nor were there any significant differences in the amount of unabsorbed dietary carbohydrate from the breakfast. (2) Using the pooled data of the total of 26 subjects, there was no significant seasonal variation in the orocecal transit time of the breakfast or the lactosucrose solution. (3) There was a significant seasonal variation in the amount of unabsorbed dietary carbohydrate from the breakfast. (4) The amount of unabsorbed dietary carbohydrate from the breakfast was largest in winter and smallest in autumn. Results in spring and in summer were similar and intermediate between those in winter and autumn. Post hoc multiple comparison tests showed that the amount of unabsorbed dietary

Submitted August 20, 2005, Returned for revision September 12, 2005, Accepted September 18, 2005

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carbohydrate in winter was significantly larger than in autumn. (5) In winter, the average ratio of the amount of unabsorbed dietary carbohydrate to the total amount of carbohydrate in the breakfast was about 12%; in autumn it was about 6%. These results clearly show that there is seasonal variation in the efficiency of intestinal dietary carbohydrate absorption among young female Japanese subjects.

Keywords Seasonal variation, Breath hydrogen test, Gastrointestinal activity, Carbohydrate maldigestion, Lactosucrose

INTRODUCTION

Many scientists have investigated the seasonality (circannual rhythm) of physiological behavior of humans. About 10 years ago, Reinberg (1997) reviewed the circannual rhythms of human nutrition. For example, Haus and colleagues (1988) found a seasonal change in the insulin response to normalized diets in normal subjects; the insulin response was strongest and fastest in autumn, and was weakest and slowest at the end of winter. Griffith found a circannual rhythm of the human Respiration Quotient (RQ); it was highest from the middle of summer to the middle of autumn, indicating that human beings metabolize carbohydrate most efficiently and predominantly in these periods of the year. Reinberg (1997) mentioned also in the same book that these two examples support the hypothesis proposed by Sargent—namely, that the seasonality of human behavior could be one of the physiological adaptations to annual environmental change, and could be referred to the behavioral rhythm of animals that hibernate. In addition, studies show that photoperiod is the most important cue for synchronizing endogenous circannual rhythms to the natural year in most circannual species (Goldman et al., 2004).

During the course of our investigation into the influence of light on the intestinal absorption of dietary carbohydrate, we obtained the following findings: (1) daytime dim-light exposure caused poorer digestion of dietary carbohydrate after the evening meal (Lee et al., 2001); (2) daytime dim-light exposure caused less absorption of dietary carbohydrate after the evening meal and less induction of gastro-myoelectric activity compared to daytime bright light exposure (Sone et al., 2003); (3) evening dim light, compared to bright light, exposure exerted a better effect on dietary carbohydrate absorption (Hirota et al., 2003). These findings were obtained experimentally under artificial light conditions but suggest an influence of natural light on seasonal efficiency of dietary carbohydrate absorption from the gastrointestinal tract. Therefore, we propose the hypothesis that there is seasonality (circannual rhythmicity) of dietary carbohydrate absorption in human beings, and here we report the results of the comparison of the amount of unabsorbed carbohydrate from the intestine of healthy Japanese subjects during each season of 2004.

In this study, we estimated the amount of unabsorbed carbohydrate by means of the breath hydrogen test. This method has become the method of choice for diagnosing the non-digestion of lactose. When lactose or any other dietary sugar is incompletely absorbed, the unabsorbed portion is fermented by colonic bacteria, forming hydrogen (as well as methane in some individuals), some of which is absorbed into the portal circulation and exhaled in the breath (Savaiano and Levitt, 2000). We describe the details of the application of this method to the present study in the following method section.

SUBJECTS AND METHODS

Subjects

Twenty-six healthy Japanese females, aged between 19 to 23 yrs, volunteered for the year-long study (over the course of the 4 seasons) as paid participants. Because the study is time-consuming (3 days of participation per subject per season) and a large number of subjects were required for statistical evaluation by analysis of variance (ANOVA), it was carried out at two universities; 12 subjects participated at Osaka City University (Osaka subgroup) and 14 subjects at Nagano Prefectural College (Nagano subgroup). All participants were healthy non-smokers. They were required to report antibiotic therapy at any time during the year (Gilat et al., 1978). All subjects underwent the breath hydrogen test during the follicular phase of their menstrual cycles, because gastrointestinal activity may vary during the cycle (Wald et al., 1981). The average age, height, and weight of each subject are listed in Table 1. We explained the purpose of the study and the procedure involved to all the subjects before they gave their written consent to participate. The conduct of the research adhered to the ethical principles and standards of the Journal (Touitou et al., 2004).

Experimental Protocol and the Breath Hydrogen Test

Figure 1 presents the experimental protocol. Subjects were requested to choose an easily digestible, low-fat evening meal for consumption at 20:00 h the day before (Day 0) and the first day (Day 1) of participation.

TABLE 1 Characteristics of Subjects

Subgroup	Number	Age (yrs)	Height (cm)	Weight (kg)
Osaka	12	21.0 ± 1.2	158.8 ± 5.2	50.0 ± 2.9
Nagano	14	19.1 ± 0.4	157.6 ± 4.8	50.6 ± 4.6

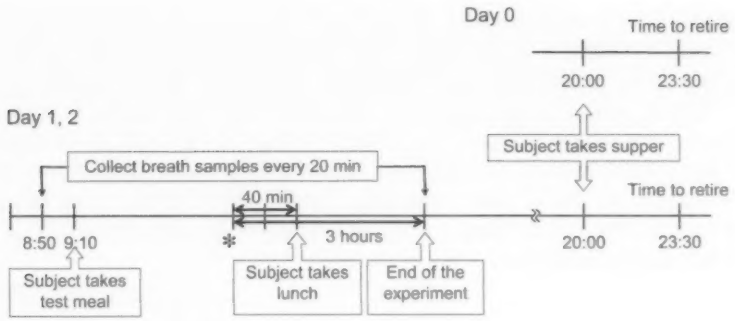

FIGURE 1 Experimental protocol (the same in the four seasons). See Figure 2 and Experiment section of the text for more detail.

Fasting periods of at least 12 h duration prior to the first breath sample were required on Days 1 and 2. Subjects were also required to retire earlier than 23:30 h on the nights of Days 0 and 1. Subjects wore a small waist accelerometer (Lifecorder, Suzuken, Nagoya, Japan) for 1 wk before their participation to record their normal daily physical activity and to be sure they adhered as closely as possible to their normal lifestyle. Analysis of the record of the 5 days of physical activities of each subject prior to their participation showed that there was no significant seasonal variation in their daily physical activities [F (3, 97) = 1.315, $p = 0.274$; one factor independent measure analysis of variance—details are in preparation for publication]. We also asked the subjects to keep a dietary record for the week before the participation using our standard dietary questionnaire (Tsumura et al., 2003).

Subjects (usually in pairs) entered the air-conditioned room at 08:30 h of Day 1 and Day 2 and remained sedentary under normal fluorescent lamps (300 to 400 lux) until the end of the breath hydrogen test, at 17:30 h. Approximate average room temperatures in Osaka and Nagano were 24°C, 25°C, 27°C, and 24°C in the winter, spring, summer, and autumn, respectively. Subjects were instructed how to collect their end-alveolar breath sample at 08:40 h, before eating a normal breakfast at 09:10 h on Day 1. This meal was prepared from commercially available ready-to-eat minestrone, boiled potato, and macaroni (nutrient composition shown in Table 2). On day 2, subjects ingested an indigestible trisaccharide solution (200 mL of water containing 6 g of commercially available lactosucrose; Ensuiko Co., Ltd. Shizuoka, Japan) at the same time as breakfast on Day 1. Every subject took the two meals in the same order in every season. Because lactosucrose is a growth factor for colonic bacteria (Ohkusa et al., 1995) and causes diarrhea in the gastrointestinal tracts of some people as a common nature of indigestive oligosaccharides, we planned for subjects to ingest the lactosucrose solution on Day 2 in

TABLE 2 Nutrient Composition of the Breakfast

Total weight	540 g
Total energy	367 kcal
Protein	12.4 g
Fat	2.5 g
Carbohydrate*	72 g
*Dietary fiber	7 g

order to avoid altering the normal microflora and physiological condition of the gastrointestinal tract during the study.

End-alveolar breath samples were collected every 20 min, beginning at 08:50 h, into special airtight bags (TERAMECS, Kyoto, Japan), and hydrogen concentrations (also methane) were measured by gas chromatography (Breath Gas Analyzer model TGA-2000, TERAMECS, Kyoto, Japan). Figure 2 shows a typical curve of hydrogen concentration *vs.* breath sampling time, where "hydrogen concentration" (ppm) is the sum of the hydrogen and methane concentrations. Because it has been shown that there is a roughly linear correlation between the excretion of hydrogen in the breath and the quantity of unabsorbed carbohydrate (Fritz et al., 1985), the area under the curve (AUC) when hydrogen concentration in the breath was plotted against time and was used to represent the amount of hydrogen due to fermentation of unabsorbed carbohydrate

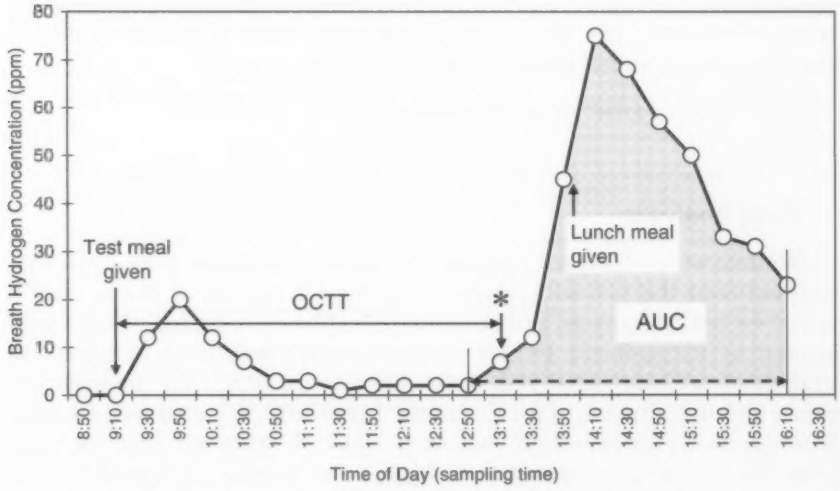

FIGURE 2 A typical relating concentration of hydrogen in the breath *vs.* time of day following breakfast. The sampling time when the head of the chyme is assumed to enter the cecum (see Experiment section of the text for more detail).

by the microflora of the large bowel. AUC was calculated according to the trapezoidal rule (Rumessen et al., 1989) and expressed in the units of parts per million hour (ppm h). We defined AUC values corresponding to the breakfast meal or lactosucrose solution as the AUC for 3 h and 20 min, commencing with the point 20 min before the rise in breath hydrogen level above the individual baseline value. This time of rise was defined as one of more than 5 ppm and one which was followed by at least two more rises (Hirakawa et al., 1988); this point is marked by an asterisk in Figures 1 and 2. At that point, the head of chyme was considered to have first entered the cecum. The length of time between 09:10 h and the time of rise (see above) was defined as the orocecal transit time (OCTT, expressed in min). Subjects ate lunch 40 min after the time of rise. The rise in the hydrogen concentration in the breath just after breakfast, as observed in Figure 2, may be due to hydrogen accumulation in the colon during sleep (Solomons et al., 1978) and/or due to fermentation of food that had not been absorbed during the previous day and had been expelled by the gastro-ileal reflex after ingestion of breakfast (Levitt et al., 1987).

The amount of unabsorbed dietary carbohydrate (UDC) from the breakfast meal was evaluated as the "lactosucrose equivalent (g)," calculated as following:

$$6 \times (\text{AUC for the breakfast meal/AUC for 6 g of lactosucrose})$$

The ratio of the UDC of the breakfast meal to its total carbohydrate content was calculated on the assumption that 7 g of dietary fiber in the breakfast meal are also fermented in the colon by its microflora (Eastwood, 1999). Therefore, the percentage of lack of intestinal digestion of the breakfast meal was calculated by the following equation:

$$\text{UDC (lactosucrose equivalent, g}/72 \times 100$$

Data Analyses

All data were expressed as means $\pm$ standard deviation (SD). Comparisons between the two subgroups (Osaka and Nagano), of mean OCTT values for both test meals, and amounts of UDC for the breakfast were performed by a two-factor (season and subgroup) independent measures analysis of variance (ANOVA). Seasonal variation in OCTT for both test meals and the amount of UDC for the breakfast were analyzed by one-factor (season) independent measures ANOVA, after pooling the data from the two subgroups, followed by *post hoc* multiple comparison tests using the Turkey HSD. We carried out the statistical analyses using SPSS ver.11.5 software package. A p value <0.05 was considered evidence of a statistically significant difference between mean values.

RESULTS

In the breath hydrogen test, the OCTT value for the breakfast indicates the length of time taken for the chyme to pass through the stomach and the small intestine, while the OCTT for the lactosucrose solution indicates the length of time taken for the oligosaccharide to pass through the small intestine only because soluble oligosaccharides are not retained in the stomach (Kondo et al., 1994). Table 3 compares the OCTT for both test meals and the UDC for the breakfast in the two subgroups. Most subject numbers in the tables are less than the number of the participants (12 in Osaka and 14 in Nagano), because some subjects in each season canceled their participation due to health problems or personal reasons. The mean OCTT values for the breakfast as well as the lactosucrose solution did not differ significantly between the two subgroups in any season [F (3, 54) = 0.258, $p = 0.855$ and F (3, 54) = 0.339, $p = 0.797$, respectively]. Analysis of the seasonal variation in OCTT for the two test meals (using pooled data) showed that there was no significant difference in OCTT for either test meal or between the four seasons [F (3, 58) = 0.839, $p = 0.478$ for the breakfast and F (3, 58) = 0.685, $p = 0.565$ for the lactosucrose solution]. The OCTTs for the breakfasts were about 70 min longer than those for the lactosucrose solution in any season (72.0 min, 72.2 min, 74.7 min, and 77.1 min in winter, spring, summer, and autumn, respectively). The one-factor (season) independent measures ANOVA showed no significant difference between the four seasons [F (3, 58) = 0.018, $p = 0.997$], indicating that the length of time during which the chyme from the breakfast was retained in the stomach was almost constant throughout the year.

The main purpose of this study was to examine the seasonal variation in UDC. We evaluated UDC as a lactosucrose equivalent value (g) because

TABLE 3 Comparison Between the Two Subgroups and at the Four Seasons of OCTT Values (min) for Both Test Meals and the Amount of UDC (g, lactosucrose equivalent) for the Breakfast

		OCTT (min)			
Subgroup	Season	Breakfast	Lactosucrose	UDC (g)	n
Osaka	Winter	231 ± 63	157 ± 35	8.1 ± 2.2	7
	Spring	229 ± 66	134 ± 50	6.9 ± 2.5	7
	Summer	226 ± 73	146 ± 71	7.7 ± 5.0	7
	Autumn	197 ± 61	130 ± 70	4.6 ± 2.3	6
Nagano	Winter	248 ± 32	178 ± 89	7.1 ± 3.2	8
	Spring	244 ± 49	185 ± 76	6.3 ± 2.8	11
	Summer	223 ± 23	153 ± 70	5.9 ± 1.5	8
	Autumn	228 ± 40	143 ± 63	4.5 ± 1.9	8

Values are expressed as mean ± SD.

the ecological population of colonic microflora may vary from season to season (Conway, 1995), and, thus, the capability of colonic microflora to ferment the undigested dietary carbohydrate may change from season to season. Therefore, we estimated the hydrogen production due to fermentation of a fixed amount of non-digestible carbohydrate (6 g of lactosucrose) by colonic microflora in each season, and then we evaluated the amount of UDC in the breakfast by comparing the AUC with that for lactosucrose solution. Figure 3 depicts UDC values as percentages for each subject (○: Osaka and ●: Nagano), and also the means (± SD) for each season. Table 3 shows the UDC values (g lactosucrose equivalent) of the two subgroups over the four seasons. The two-factor (season and subgroup) independent measures ANOVA showed there was no significant difference in the amount of UDC between the two subgroups in any season [F (3, 54) = 0.213, $p = 0.887$]. Accordingly, we pooled the data and analyzed the UDC for each season (Table 4). This table shows that there was a significant seasonal change in the amount of UDC [F (3, 58) = 3.134, $p = 0.032$], and the *post hoc* multiple comparison by the Turkey HSD indicated that there was a significant difference between winter and autumn ($p = 0.022$).

DISCUSSION

We have found no difference in the mean values of the parameters of gastrointestinal activity, such as orocecal transit time (OCTT) of the test meals and the amount of unabsorbed dietary carbohydrate (UDC) from

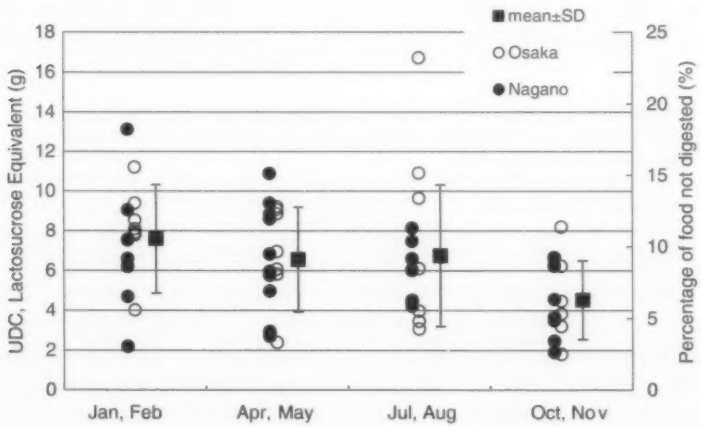

FIGURE 3 Amount of UDC (g lactosucrose equivalent) and percentage of non-digestion per subject after ingestion of the breakfast (○: Osaka and ●: Nagano), means of UDC (with SD bars) shown for the four seasons.

TABLE 4 Summary Table for One-Factor (Season) Independent Measures ANOVA for the Amount of UDC in Four Seasons

	Sum of squares	*df*	Mean square	*F*	*p*
Between groups	72.92	3	24.305	3.134	0.032
Within groups	449.81	58	7.755		
Total	522.72	61			

the breakfasts, between the two groups of subjects in any season. Geographically, the cities of Osaka and Nagano are situated relatively close each other; Osaka is situated about 2° south and 2° west from Nagano (in 34° 41 N, 135° 31 E and in 36° 40 N, 137° 12 E, respectively). Also, seasonal changes in sunshine duration of the two cities are very similar (National Astronomical Observatory, 2005). These similarities might explain the lack of difference in the gastrointestinal activity of the subjects of the two subgroups through out the year. As a result, the data obtained from the two subgroups were pooled.

We did not find significant seasonality in the OCTT of the two test meals. This result indicates that the time taken for the meal to pass through the stomach and the small intestine—*i.e.*, the gastrointestinal motility that propels ingested food toward the cecum—did not change with the season. In addition, the constant length of time taken for the stomach to empty the chyme indicates that the gut's mechanical activities were also constant throughout the year. In relation to this mechanical activity of the stomach, we recorded each subject's electrogastrography (EGG) for the two test meals, and we are now analyzing these data to determine if seasonality exists in the electrical activity of the stomach.

In contrast, we found significant seasonal variation in the amount of UDC in the standard breakfast; there were significant differences in the rate of gastrointestinal digestion and in the absorption of dietary carbohydrate. The absorption of carbohydrate was best in autumn and worst in winter. To discuss the physiological cause that is responsible for this phenomenon, we first need to refer to the everyday life factors that may influence the digestion of dietary carbohydrate, such as the physical activities of the subjects. Textbooks on physiology (*e.g.*, Ganong, 1999) state that gastrointestinal activity is regulated by the autonomic nervous system, and that increased sympathetic nervous system activity reduces the rate of nutrient absorption in the gastrointestinal tract and that the tone of the autonomic nervous system is closely related to the subject's physical activities. Therefore, seasonal variation in the tone of autonomic nervous system and function as well as the subject's physical activities should be considered. The seasonality of autonomic nervous system tone was explored previously by Kazuma and colleagues (2000); they reported seasonal variation in heart rate variability of healthy Japanese children aged 5 to 15 yrs

but found that no seasonal variation in the high-frequency (HF) band (0.15 to 0.4 Hz), the low-frequency (LF) band (0.04 to 0.15 Hz), or LF/HF. As described in the Methods section of this paper, we recorded the subjects' physical activities in each season using a small wrist accelerometer. There was no significant seasonal variation in the subjects' daily physical activities. This result suggests it is not necessary to consider the effects of seasonal changes in the autonomic nervous system and subject physical activities on the seasonal changes in gastrointestinal activity and absorption of dietary carbohydrate. Constant OCTT values for the test meals obtained in this study also support the view that there was no significant variation in gastrointestinal activity throughout the year.

In opposition to the above discussion suggesting no influence of the autonomic nervous system on the seasonal variation of gastrointestinal activity, the following hypothesis is offered to explain why the absorption of carbohydrate was found to be worst in winter. In winter, subjects are exposed to longer duration of dim light. The sympathetic nervous system would be expected to be more activated under such conditions, evidence for this being the finding that systolic and diastolic blood pressure are increased significantly under longer dim light exposure (Kim and Tokura, 2000). Furthermore, Kanikowska and coworkers (2002) showed that salivary secretion was significantly lower under morning dim-light exposure, suggesting less activity of the parasympathetic nervous system innervating the salivary glands. Therefore, we suggest that it is probable that longer dim light exposure made digestive activity worse in winter by increasing sympathetic nervous activity. Temperature must also be taken into account. Generally speaking, lower ambient temperature excites the sympathetic nervous system (Ganong, 1999), as evidenced for example by stronger vasoconstriction in winter (Aschoff, 1971), resulting in reduced intestinal activity.

We can only speculate why the efficiency of the intestinal absorption of dietary carbohydrate is better in the autumn than winter. The physiological relationship between the present findings and the circannual rhythms of human respiratory quotient (RQ) and insulin response, which are directly related to human carbohydrate metabolism, is relevant. The circannual rhythm of RQ is highest in the middle of summer than middle of autumn (Reinberg, 1997), and this is consistent with our finding that humans absorb dietary carbohydrate most efficiently in autumn and metabolize carbohydrate most efficiently and predominantly at these times of the year. Haus and associates (1988) described the circannual changes in the circadian variation in plasma insulin in clinically healthy women. The response of insulin to three meals varied as a function of the season and, during the same season, as a function of the circadian stage. In winter and spring, the response to the three meals appeared to be similar, while in summer and autumn, the response appeared to be

higher. The figure in their review shows that the insulin response to the three meals was in the order: autumn > summer > spring > winter. Although we carried out the current investigation only on breakfasts, our result is consistent with these observations on insulin response. Moreover, our finding could explain the circannual changes in human insulin response; the more the intestine absorbs dietary carbohydrate in autumn, the more the blood sugar levels rise after a meal, and so more insulin is released into the circulation. Therefore, our present result—that there is a seasonal variation in the efficiency of the intestinal absorption of dietary carbohydrate—is an intrinsic physiological response to annual environmental change, and supports the hypothesis proposed by Sargent as described in the Introduction.

Finally, we calculated the ratio of the UDC of the breakfast to its total carbohydrate content on the assumption that most carbohydrates in the breakfast are fermented in the colon by its microflora. Figure 3 also illustrates the seasonal change in the lack of digestion of the breakfasts, with about 12% of total carbohydrate escaping digestion and absorption by the small intestine in the winter in comparison to only about 6% in the autumn. If such a low efficiency of carbohydrate absorption occurs in all three meals in winter, our finding could explain one of the symptoms in patients with winter Seasonal Affective Disorder (SAD), *i.e.*, their increased appetite for sweet foods and carbohydrate craving (Partonen and Rosenthal, 2001). In this connection, we have carried out a similar study in Poznan, Poland, where the difference in the light environment between the four seasons is much greater than in Japan. Some of these results were presented at the Russian-Japanese Symposium on physiological anthropology (Tsumura et al., 2005). The detailed results obtained in the Polish study, and the comparison of seasonal variation of UDC between Japanese and Polish subjects, will be published elsewhere.

ACKNOWLEDGMENTS

The authors are very grateful to Ms. Kuniko Tsukamoto and Sakiko Sugiyama for their technical assistants. The authors thank sincerely Dr. J. Waterhouse, United Kingdom, for his critical reading and editional assistance of the manuscript. This work was supported by Grants-in Aid for Scientific Research (No. 15207024) from Japan Society for the Promotion of Science.

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Chronobiology International, 22(6): 1121–1135, (2005)
Copyright © 2005 Taylor & Francis LLC
ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398106

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SEASONAL VARIATION IN ONSET OF MYOCARDIAL INFARCTION—A 7-YEAR SINGLE-CENTER STUDY IN ITALY

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Like many other serious acute cardiovascular and cerebrovascular events, acute myocardial infarction (AMI) shows seasonal variation, being most frequent in the winter. We sought to investigate whether age, gender, and hypertension influence this pattern. We studied 4014 (2259 male and 1755 female) consecutive patients with AMI presenting to St. Anna Hospital of Ferrara, Italy between January 1998 and December 2004. Some 1131 (28.2%) of the AMI occurred in persons <65 yrs of age, and 2883 (71.8%) in those ≥65 yrs of age. AMI was over-represented in males (82% in the <65 yr group *vs.* 56.6% in the ≥65 yr group ($\chi^2 = 13.99$; $p < 0.001$). Hypertension had been previously documented in 964 (24%) of the cases. There were 691 (17.2%) fatal case outcomes; fatal outcomes were significantly higher among the 3054 normotensive ($n = 614$ or 20.1%) than the 964 hypertensive cases ($n = 77$ or 8%; $\chi^2 = 74.94$, $p < 0.001$). AMIs were most frequent in the winter ($n = 1076$ or 26.8% of all the events) and least in the summer ($n = 924$ or 23.0% of all the events; $\chi^2 = 12.36$, $p = 0.007$). The greatest number of AMIs occurred in December ($n = 379$ or 9.44%), and the lowest number in September ($n = 293$ or 7.3%; $\chi^2 = 11.1$, $p = 0.001$). Inferential chronobiological (Cosinor) analysis identified a significant annual pattern in AMI in those ≥65 yrs of age, with a peak between December and February—January for the total sample ($p < 0.005$), January for the sample of males ($p = 0.014$), February for fatal infarctions ($p = 0.017$), and December

Submitted July 6, 2005, Returned for revision August 25, 2005, Accepted September 2, 2005
Supported by a scientific grant of the University of Ferrara, Italy.
Data presented, in part, at the EFIM 2005 (The European Federation of Internal Medicine Congress), Paris, August 31 to September 3, 2005.
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for non-fatal infarctions ($p = 0.006$). No such temporal variations were detected in any of these categories in those <65 yrs of age. The annual pattern in AMI was also verified by Cosinor analysis in the following hypertensive subgroups: hypertensive males ($n = 552$: January, $p = 0.014$), non-fatal infarctions in hypertensive patients ($n = 887$: January, $p = 0.018$), and elderly normotensives ($n = 1556$: November, $p = 0.007$).

Keywords Acute myocardial infarction, Annual variation, Seasonal variation, Chronobiology, Age, Gender, Hypertension

INTRODUCTION

Speculation on the effects of climate upon health dates back at least to Hippocrates and his "Air, Waters and Places." A large body of evidence indicates that the manifestation of serious cardiovascular and cerebrovascular events does not occur randomly throughout the year. Each displays annual variation, with peak incidence in the winter. Earlier studies, in fact, revealed an inverse and approximately linear relationship between ambient temperature and coronary heart disease as well as stroke mortality in selected areas of the United States (Rogot and Padgett, 1976). More recently, this winter preference has been confirmed for ischemic and hemorrhagic stroke (Gallerani et al., 1993, 1996; Manfredini et al., 2005), pulmonary embolism (Gallerani et al., 1992; Manfredini et al., 1994, 2004a), aortic dissection (Mehta et al., 2002, 2005a, 2005b), sudden death (Arnt et al., 2000), and heart failure hospitalization and death (Boulay et al., 1999; Stewart et al., 2002). Moreover, it has been reported recently that the number of deaths in patients with congestive heart failure (CHF) after prior myocardial infarction is significantly higher during the cold winter and warm summer months than during the more temperate spring and fall months (Aronow and Ahn, 2004).

Acute myocardial infarction (AMI) has been widely investigated as well, with most studies identifying a winter peak and a summer nadir. Data from the U.S. National Registry of Myocardial Infarction (NMRI) (Spencer et al., 1998) reported the occurrence of ≈53% more cases in winter than summer, with a peak in-hospital case fatality rate of 9% in the winter. Interestingly, the winter peak has been reported not only for first-time AMIs, but also for second-occurrence AMIs (Figueras et al., 2002). A number of studies conducted in different locations around the world have confirmed the winter preference (see Figure 3); although, some studies conducted in geographical regions lacking significant temperature extremes have not always documented seasonal variation in AMI (Chang-Sheng et al., 1988). We sought to investigate the seasonal pattern of presentation of AMI in Ferrara, Italy, and to explore the possible influences of age, gender, and previous diagnosis of hypertension on it.

SUBJECTS AND METHODS

The study was conducted with the approval of the local institutional committees for human research and in accord with the guidelines of the Journal (Touitou et al., 2004). All consecutive subjects with AMI presenting to St. Anna Hospital of Ferrara, Italy, during the 7 yr span between January 1998 and December 2004 were included. Ferrara is located in northeastern Italy and has a population of 150,000; its demographic and socioeconomic characteristics are similar to those of Italy as a whole. The population is almost exclusively white. The only hospital is St. Anna Hospital, serving also the University of Ferrara, and the distribution of the ambulance starting points throughout the territory allows an average time of arrival to the Emergency Department (ED) $\leq$15 min from the time a call for emergency service is received.

The diagnosis of AMI was made on the basis of symptoms consistent with acute myocardial ischemia (chest pain, dyspnea, cardiac shock, cardiac arrest), qualifying electrocardiographic (EKG) changes in consecutive leads (ST elevation or depression of at least 1 mm, new T-wave inversion of at least 1 mm, pseudo-normalization of previously inverted T-waves, new Q-waves, new R-wave greater than S-wave in V_1, or new left bundle branch block), positive cardiac enzymes (CK-MB or troponin above the upper limit of normal), and tissue necropsy. Symptom onset was defined as the date (day, month, and year) that a patient, relative, or witness first noted definite symptoms or signs (chest pain, dyspnea, shock, or cardiac arrest). Presence of hypertension was established by medical history, general practitioner, hospital charts, and use of antihypertensive drugs.

Day of symptom onset was categorized both into 12 1-month intervals and into four 3-month intervals (Spring: March 21 to June 20, Summer: June 21 to September 22, Fall: September 23 to December 20; Winter: December 21 to March 20) for analysis. The distribution of symptom onset within the four 3-month periods was tested for uniformity in the overall population and in the various case subsets by the χ^2 test for goodness of fit (Snedecor, 1978). Chronobiological analysis of annual variation in AMI was performed by application of partial Fourier analysis to the time series data using Chronolab software written for the Apple Macintosh computer (Mojòn et al., 1992). This method selects the harmonic, or the combination of harmonics, that best explains the variance of the time series data. The percentage of the overall variability in the data about the arithmetic mean attributable to the fitted cyclic function is an estimator of the goodness of fit of the given approximation. The F-test statistic was used to test the zero-amplitude null hypothesis (absence of cyclic variation). The parameters derived from the overall 1 yr (8766 h) period cosine approximation of

the time series data were the midline estimated statistic of rhythm (MESOR, the rhythm-adjusted mean over the time period analyzed), amplitude (half the distance between the absolute maximum and minimum of the fitted curve), and the peak and trough time (time of occurrence of absolute maximum and minimum). Significance levels were set at $p < 0.05$.

RESULTS

The sample consisted of 4014 (2259 male and 1455 female) cases of AMI representing a mean age of 71.1 ± 12.6 yrs (males: 68.2 ± 12.5 yrs and females: 76.3 ± 11 yrs). There were 3544 Q-wave and 470 non–Q-wave infarctions. Some 1131 (28.2%) of the AMI occurred in those <65 yrs, and 2883 (71.8%) occurred in those ≥65 yrs. Infarctions was more represented in males (82% in the <65 yr group and 56.6% in the ≥65 yr group; $\chi^2 = 13.99$, $p < 0.001$). The demographic features of the total population are reported in Table 1a and 1b.

Some 691 or 17.2% (of the total 4014) cases had a fatal course, 674 Q-wave and 17 non–Q-wave infarctions, respectively. Hypertension was documented in 964 of the cases, 24% of the entire sample of cases. There was a significantly higher number of fatal infarctions among the 3050 normotensive than the 964 hypertensive cases (614/3050 or 20.1% *vs.* 77/964 or 8% fatality, respectively; $\chi^2 = 74.94$, $p < 0.001$).

The highest number of AMI occurred in the winter ($n = 1076$ or 26.8%) and the lowest number in summer ($n = 924$ or 23.0%) as shown in Figure 1; the seasonal difference in the number of cases was statistically significant ($\chi^2 = 12.36$, $p = 0.007$). Examination of the monthly data (Figure 2) revealed the peak number of AMI occurred in December ($n = 379$ or 9.44%) and the nadir in September ($n = 293$ or 7.3%), the difference being statistically significant ($\chi^2 = 11.1$, $p = 0.001$).

Inferential chronobiological analysis failed to identify a seasonal temporal pattern in AMI in those <65 yrs (Table 2a); whereas, such was identified in older cases with a peak: in January for the total sample ($p < 0.005$), January for males ($p = 0.014$), February for fatal infarctions

TABLE 1a Acute Myocardial Infarctions (AMI), 1998–2004, Hospital of Ferrara, Italy. Demographic Features (I)

AMI	*n* (%)	<65 (%)	>65 (%)
All	4014	1131	2883
Males	2559 (63.8)	927 (82.0)	1632 (56.6)
Females	1455 (36.2)	204 (18.0)	1251 (43.4)

TABLE 1b Acute Myocardial Infarctions (AMI), 1998–2004, Hospital of Ferrara, Italy. Demographic Features (II)

AMI	*n* (%)	Q-wave (%)	non–Q-wave (%)
All	4014	3544	470
Fatal	691 (17.2)	674 (19.0)	17 (3.6)
Non-fatal	3323 (82.8)	2870 (81.0)	453 (96.4)
Hypertensives			
All	964	834	130
Fatal	77 (8.0)	72 (8.6)	5 (3.8)
Non-fatal	887 (92.0)	762 (91.4)	125 (96.2)
Normotensives			
All	3050	2710	340
Fatal	614 (20.1)	602 (22.2)	12 (3.5)
Non-fatal	2436 (79.9)	2108 (77.8)	328 (96.5)

($p = 0.017$), and December for non-fatal infarctions ($p = 0.006$). The presence/absence of the risk factor of hypertension also played a role (Table 2b). The seasonal pattern was found only for hypertensive males ($n = 552$: January, $p = 0.014$), non-fatal infarctions in hypertensive subjects ($n = 887$: January, $p = 0.018$), and elderly normotensive subjects ($n = 1556$: November, $p = 0.007$).

FIGURE 1 Geographic location and results of several studies on acute myocardial infarction done around the world.

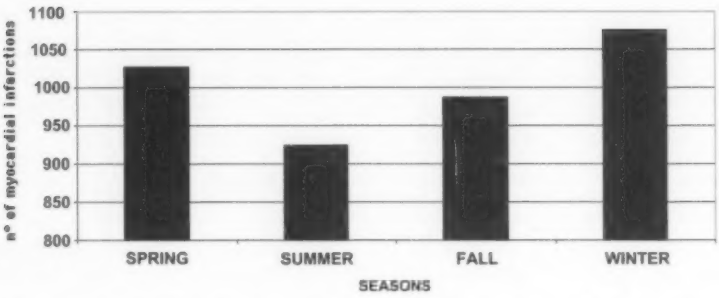

FIGURE 2 Seasonal distribution of acute myocardial infarctions in Ferrara, Italy, 1998–2004.

DISCUSSION

Seasonal variation in cardiovascular morbidity and mortality has been investigated in several countries with differing climatic conditions. Figure 3 identifies the geographic location of studies done around the world. Although some authors have also suggested the participation of a series of factors, *e.g.*, respiratory infections (Woodhouse et al., 1994), air pollution (Rossi et al., 1999), and solar radiation (Scragg et al., 1990), winter season and cold weather seem to be common and important factors.

The results of the present study confirmed the seasonal pattern of AMI onset and its peak in winter and trough in summer, especially in the elderly. Hypertension does not seem to significantly affect this seasonal pattern. Interestingly in our cohort of patients, fatal myocardial infarctions

TABLE 2a Results of Chronobiologic Analysis for Annual Patterning of AMI: Subgroups by Age.

Age group	Category	*n*	Peak	95% CL	*p* Value
All	Males	2559	Jan	Dec/Feb	0.014
	Females	1455	—	—	ns
	Fatal	691	Feb	Jan/Mar	0.012
	Non-fatal	3323	Dec	Nov/Jan	0.006
	All	4014	Jan	Dec/Feb	0.005
Age < 65 yr	Males	927	—	—	ns
	Females	204	—	—	ns
	All	1131	—	—	ns
	Fatal	43	—	—	ns
	Non-fatal	1088	—	—	ns
Age ≥ 65 yr	Males	1632	—	—	ns
	Females	1251	—	—	ns
	All	2883	Jan	Dec/Feb	0.004
	Fatal	648	Feb	Jan/Mar	0.017
	Non-fatal	2235	Dec	Nov/Jan	0.006

95% CL = 95% confidence level.
ns = not significant.

TABLE 2b Results of Chronobiologic Analysis for Annual Patterning of: Subgroups by Presence or Absence of Hypertension.

Group	Category	*n*	Peak	95% CL	*p*-value
All	Males	2559	Jan	Dec/Feb	0.014
	Females	1455	—	—	ns
	Fatal	691	Feb	Jan/Mar	0.012
	Non-fatal	3323	Dec	Nov/Jan	0.006
	All	4014	Jan	Dec/Feb	0.005
Hypertensives	Males	552	Jan	Dec/Feb	0.014
	Females	412	—	—	ns
	All	1064			
	Fatal	77	—	—	ns
	Non-fatal	887	—	—	ns
Normotensives	Males	2007	—	—	ns
	Females	1043	—	—	ns
	All	3050			
	Fatal	614	—	—	ns
	Non-fatal	2436	—	—	ns

95% CL = 95% confidence level.
ns = not significant.

were significantly more frequent among normotensive than hypertensive persons. Recent studies have reported a similar finding. In fact, Majahalme and colleagues (2003) showed in a study of a consecutive cohort of subjects with a discharge diagnosis of acute coronary syndrome (ACS) that hypertensives had fewer incidents of AMI than did normotensives; moreover, AMI seemed to be related with a higher frequency of percutaneous coronary intervention and coronary artery bypass grafting.

With some exceptions of findings from warm climate countries (Heyer et al., 1953; De Pasquale et al., 1961; Freeman et al., 1976; Kolar et al.,

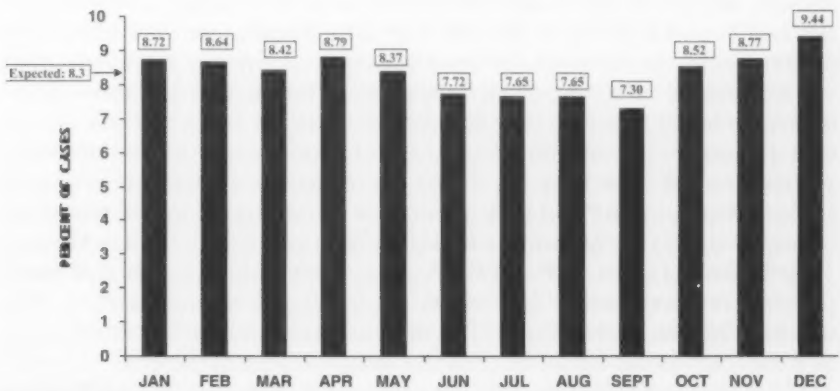

FIGURE 3 Monthly distribution of acute myocardial infarctions in Ferrara, Italy, 1998–2004.

1998), the majority of studies have reported the lowest frequency of AMI onset in the summer. In addition, it has been reported that both non-reperfused and reperfused myocardial infarctions are smallest in size when occurring in the summer (Kloner et al., 2001). Theoretically, the warm summer ambient temperature might contribute to reduced vascular resistances. Thus, both preload and afterload by venous and arterial dilation, respectively, could be reduced, with the final result being reduced left ventricular wall stress and consequent reduced myocardial oxygen demand.

Most studies have identified winter as the most vulnerable period of the year for AMI. There are several possible mechanisms by which winter may constitute a high risk period for AMI onset. In fact, a cluster of pathophysiologic factors, such as arterial blood pressure (BP), plasma viscosity, coagulation factors, and lipid status, which may contribute to an increased tendency toward arterial thrombosis, exhibits significant seasonal variation with a winter-time peak.

Arterial Blood Pressure

BP is a strong risk factor for AMI. The association between change in BP and change in temperature has been widely investigated, and a significant negative correlation between ambient temperature and BP, especially in climates with cold winters, has been demonstrated (Kunes et al., 1991). The increased level in BP in winter seems to result from several factors, including increased sympathetic activity and decreased loss of fluids and sodium (Sharma et al., 1990). Although some negative reports exist (Brueren et al. 1998), the majority of studies have reported a seasonal pattern or influence on arterial BP. Brennan and associates (1982), in their large population study on British subjects with mild hypertension, showed mean summer-winter differences of 6 to 7 mm Hg in systolic (S) BP and 3 to 4 mm Hg in diastolic (D) BP. Woodhouse and coworkers (1993) found the seasonal variation in BP to be greater in elderly than young subjects. This group of investigators found the variation in the elderly to be 13.2 mm Hg for SBP and 7 mm Hg for DBP, with no significant differences between normotensive and hypertensive subjects. A recent Israeli study (Charach et al., 2004) confirmed in elderly hypertensive subjects that both SBP and DBP are highest during the winter, with significant ($p < 0.001$) mean winter-summer differences of 31 ± 19 mm Hg and 15 ± 14.3 mm Hg for SBP and DBP, respectively. Moreover, 38% of study patients required more aggressive antihypertensive treatment in the winter. The study also found that myocardial infarction occurred twice as frequently in the winter than in the other seasons of the year.

The seasonal differences in BP reported by Charach and colleagues (2004) were much higher than those reported by Woodhouse and

associates (1993). The authors of the Israeli study point out that the subjects studied by Woodhouse and coworkers (1993) had mild hypertension, and they speculate that more severely hypertensive patients perhaps show different responses to environmental temperature and other factors. More recently, after adjustment for body mass index, age, sex, baseline SBP, and clinical center, Jehn and associates (2002) showed that SBP variability was inversely associated with both the mean 24 h as well as daytime ambient temperature.

The seasonal variation in BP may have implications in determining a condition of increased cardiovascular risk. A large meta-analysis of prospective data (MacMahon et al., 1990) showed that a 5 mm Hg increase in DBP was associated with a 21% increase in risk of coronary heart disease (CHD) and a 34% in risk of stroke. Based on the findings of this study as well as the findings of other studies relating changes in BP to change in ambient temperature, a 5°C decline in ambient temperature during the winter would be expected to result in a 4% increased in risk of a CHD event and an 8% increased risk of stroke (Chang et al., 2004). However, data from the WHO MONICA Project (Enquselassie et al., 1993) showed that coronary events, both fatal and non-fatal, were 20% to 40% more likely to occur in winter and spring than other times of the year; moreover, coronary deaths were more likely to occur on days characterized by low environmental temperatures.

Coagulation

Keatinge and colleagues (1984) demonstrated a series of changes that could account for an increased risk of thrombosis in the cold during normal thermoregulatory adjustments. A mild surface cooling, in fact, even with core body temperature in the normal range and well above hypothermic values, increases the packed cell volume (+7%), platelet count, and the mean platelet volume to produce a final increase in the fraction of plasma occupied by platelets (+15%), and consequently whole blood viscosity (+21%). Moreover, several studies have reported the existence of a stable and predictable seasonal variation in fibrinogen levels. This variation has been estimated to be 13 mg/dL, 31 mg/dL, 40 mg/dL, and 71 mg/dL, respectively, in studies conducted, respectively, by Woodhouse and associates (1994), van der Bom and colleagues (1997), Hermida and coworkers (2003), and Stout and Crawford (1991). A previous epidemiologic study (Meade et al., 1986) reported that an increase in plasma fibrinogen concentration of 59 mg/dL was associated with an increased risk of death from ischemic heart disease by 67% in the first 5 yr of follow-up. Based on this association, Hermida and associates (2003) estimated the seasonal difference in plasma fibrinogen of 40 mg/dL, as found in their study conducted in Spain, should be associated with a 45% increase in the risk of

ischemic cardiac mortality in the winter time. Synchrony between the peak size of platelets and the peak level of fibrinogen has been reported by Crawford and colleagues (2003a, 2003b), and this might result in increased likelihood of thrombotic events in the winter. Although some authors suggested that the mechanism underlying this seasonal increase in fibrinogen levels is activation of the acute phase response secondary to winter respiratory infections (Woodhouse et al., 1994), further studies have not confirmed this hypothesis (Horan et al., 2001). Recently, it has been shown that hypertensive subjects exhibit a highly significant seasonal variation in plasma fibrinogen with a peak in winter and that non-dipper hypertensives (BP decline during nocturnal sleep less than 10% of the daytime mean level) have higher plasma fibrinogen levels than dippers (BP decline 10% to 20% of daytime mean level) throughout the year (Hermida et al., 2003).

Lipids

Several studies have reported higher cholesterol levels in the winter than the other seasons of the year. Results of the Lipid Research Clinics Coronary Primary Prevention Trial Placebo Group (Gordon et al., 1988) showed highly significant seasonal cycles, with a peak in winter for the plasma levels of total cholesterol (TOT-C), low-density lipoprotein (LDL-C), and high-density lipoprotein (HDL-C), characterized by mean changes of 7.4, 6.4, and 0.8 mg/dL, respectively. No significant variation was found in triglycerides. In an elderly population, Woodhouse and colleagues (1993a) confirmed the seasonal variation in serum total cholesterol (0.32 mmol/L, $p < 0.001$), while HDL-C was higher in winter only in males (0.16 mmol/L, $p < 0.001$) and triglycerides only in women (0.22 mmol/L, $p = 0.002$). The annual rhythm of blood cholesterol has been confirmed to be independent of age, gender, BMI, overall diet, or physical activity (Blüher et al., 2001). The observed seasonal difference between the maximum and minimum concentrations amounted to about 5% to 10% for TOT-C and 5% to 8% for HDL-C. Ockene and coworkers (2004) also reported seasonal changes in cholesterol with the peak in winter and trough in summer; however, they found TOT-C and HDL-C to be of lower amplitude, 1.8% and 2.5% of the annual average cholesterol levels, respectively. Nonetheless, we believe that higher cholesterol levels should be added to the constellation of risk factors that are most pronounced in winter (Manfredini et al., 2004b).

Endogenous Factors

It is also possible that the seasonal variations in cardiovascular functions are related to factors other than environmental temperature, and

that endogenous rhythms may play an important role as well. In fact, genetically determined endogenous rhythms may mediate seasonal variations in cardiovascular functions, in relation to endocrine and/or metabolic functions (Hata et al., 1982; Nicolau and Haus, 1989). Moreover, endogenous rhythms may be affected by ambient factors that show seasonality beyond ambient temperature, *e.g.*, photoperiod, which might in themselves synchronize endogenous annual rhythms in parameters that constitute primary or secondary risk factors of AMI (Haus et al., 1987). Indeed, the annual variation in arterial BP, coagulation, and lipids, as presented above, might be expressions of endogenous circannual rhythms that might be enhanced or suppressed by seasonal changes in ambient temperature, physical activity, emotional stress, and food consumption/eating habits, among other factors. It is difficult to identify the origin (*i.e.*, whether it be endogenous or exogenous) of the observed annual variation in risk factor parameters of AMI and/or the degree to which endogenous rhythms are positively or negatively masked by the external environmental and life habits.

CONCLUSIONS

The risk of AMI is greatest in the winter, especially in the elderly. Several studies indicate that elderly subjects are less able to regulate body temperature (Florez-Duquet and McDonald, 1998) and seem to have less capacity to protect either their internal temperature or their extremities against cold temperatures. Collins and coworkers, (1995) showed that cold exposure for some hours caused elderly (but not young) subjects to undergo a drop in body temperature by 0.4°C and undergo an increase in BP (greater in the elderly than younger subjects). Woodhouse and colleagues (1993b) found that every 1°C increment in living room temperature to be associated with a 1.3/0.6 mm Hg reduction in SBP and DBP in the elderly, while a 1°C increment in mean outdoor temperature was associated with a 0.8/0.3 mm Hg reduction in SBP and DBP. In this regard, Keatinge and associates, (1984) recommend that indoor room temperature be kept comfortable during the cold winter months. Danet and colleagues, (1999) further recommend better protection against cold weather, through active heating, good residential insulation, and use of adequate clothing, especially for those at risk to coronary artery disease. Interestingly, a significant reduction of the rate-pressure index (SBP × heart rate) has been demonstrated in subjects adapted to cold (De Lorenzo et al., 1999), suggesting that decreased sympathetic activity and serum lipid levels, plus changes in hemostatic factors during short and intermittent adaptation to cold, might reduce the risk of ischemic heart attack in winter (De Lorenzo et al., 1999).

Aggressive clinical management of winter-time BP increase with antihypertensive medications in those exhibiting marked seasonal changes is seemingly an important preventive measure (Rosenthal, 2004). Moreover, aggressive clinical monitoring and management of seasonal variations in cholesterol, coagulation, eating habits, and cardio-conditioning, with added emphasis on patient compliance, constitute additional key preventive measures, particularly for the elderly and other susceptible persons at elevated risk of winter-time events.

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Chronobiology International, 22(6): 1137–1143, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398114

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SHORT COMMUNICATION

INFLUENCE OF LUNAR PHASES ON SUICIDE: THE END OF A MYTH? A POPULATION-BASED STUDY

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The hypothesis of lunar influence on suicide remains widespread, despite the fact that little scientific evidence to substantiate it. We conducted a population-based study to assess the influence of the lunar phases on suicides according to age, sex, and chosen method. The study included all suicides in Middle Franconia between 1998 and 2003. From a population-based sample of 3351 events, the files of 3054 suicides (1949 males and 1105 females) were complete for the study variables. Data were categorized by lunar phase, sex, age, and chosen method—"violent" *vs.* "non-violent" acts. No significant relationship was detected between the full, absent, and moon's interphases and suicide incidence. Nevertheless, there was a weak association between the absent moon and choice of a non-violent suicide method in men aged less than the median of 40.2 yrs. There was no evidence of a relationship between suicide and lunar phase. Some explanations for this phenomenon are discussed.

Keywords Suicide, Lunar phases, Lunar rhythm, Population-based study

INTRODUCTION

Temporal patterns of suicides have been of interest to many authors over the years. The hypothesis of cosmic influence on human behavior, especially the occurrence of suicide, has been entertained for more than 2,500 yrs, since Hippocrates explained that "one who is seized with terror, fright, and madness during the night is being visited by the goddess of the moon" (cited in Jones and Jones, 1977). Contrary to the popular notion that suicide occurrence is influenced by lunar phases, especially the full moon, most studies have failed to confirm this

Submitted June 6, 2005, Returned for revision August 8, 2005, Accepted August 29, 2005
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common belief (Garth and Lester, 1978; Jones and Jones, 1977; Lieber, 1975; MacMahon, 1983; Martin et al., 1992; Pokorny, 1964). Only a small number of studies have reported evidence of a significant influence of the full moon on the risk for suicide (Jones and Jones, 1977; Yvonneau, 1996). In this respect, it is speculated that "biological tides" (Lieber and Sherin, 1972) or changes in weather via lunar influences might contribute to the association of the moon's influence on the timing of suicidal acts (Jones and Jones, 1978).

Nevertheless, the continued prevalence of the belief in the effect of the moon on suicide and human behavior, in general, could have profound consequences on people dealing with suicide prevention or investigating unnatural deaths (Vance, 1995). Associations between lunar phases and suicide might differ by region and over time. The relationship between lunar phases and suicide has not previously been examined in Germany. Therefore, we decided to explore the influence of lunar phases on suicide with regard to age, sex, and the chosen method utilizing a population-based sample of suicides in Middle Franconia (Bavaria, Germany) that occurred between 1998 and 2003. The advantage of this study lies in the accuracy of the timing of death, the number of suicides, and the completeness of the database of the study population. Furthermore, to our knowledge, the influence of lunar phases on both age and sex, in relation to the chosen method, (*i.e.*, violent or non-violent method of suicide) had not been previously studied.

MATERIALS AND METHODS

Samples

The study included all residents of Middle Franconia, who died between January 1, 1998 and December 31, 2003 and whose death was classified as a suicide by the death certificate and police investigations. Middle Franconia has about 1.7 million inhabitants. The study involved the data of 3054 (1949 male and 1105 female) suicides obtained from a population-based sample of 3351 suicides. Cases with unknown exact times of death and missing data concerning the chosen method of suicide were excluded.

The study was conducted following the ethical guidelines recommended by the Journal (Touitou et al., 2004). For each suicide case, information on sex and exact time and date of death was extracted from the files of the police department of Middle Franconia as acquired by detailed enquiries of the criminal investigation department. Due to the obligations of secrecy, data concerning occupation, marital status, and impairment through alcohol, drugs, and medical history were not available.

The stage of the lunar cycle at the time of each suicide death was identified from the date and time of death of each case following the calendar for the years 1998 to 2003. Cases with unknown times of death were excluded. Suicides were categorized into three groups. The number of suicides in a 2-day period (± 48 h) centered on the full moon and the number of suicides in a 2-day period (± 48 h) centered on the absent moon were compared to the number of suicides occurring during the remainder of the lunar cycle.

Suicides were divided with regard to sex as well as the chosen, *i.e.*, "violent" *vs.* "non-violent" method of suicide. "Violent" methods included hanging, drowning, shooting, vehicle accidents, and stabbing; "non-violent" methods included death by poisons or gas. Furthermore, data were categorized with regard to age, younger or older than the median age of the sample, 40.2 yrs.

Statistical Methods

All statistical tests were two-sided. The significance level was set at $\alpha = 0.05$. Univariate analysis was performed by applying the χ^2-test statistic and by calculating suicide rates in relation to the length of each moon phase with each lunar cycle lasting 29.4 days, as described in the legend of each table. The association was evaluated for the potential influence of confounding factors, such as day of week and month as well as season of the year. All calculations were performed using the statistical package SPSS™ (Chicago, IL, USA) 10 for Windows.

RESULTS

The distribution of 3054 suicides during the full or absent moon and the other lunar phases did not differ significantly from the expected values based on the assumption that the occurrence of suicide is uniform over time (Tables 1 and 2). Also, no gender related differences were observed in suicide over the lunar cycle (Figure 1). Furthermore, there were no weekly ($\chi^2 = 12.7$, $df = 12$, $p = 0.392$), monthly ($\chi^2 = 19.1$, $df = 22$, $p = 0.640$), or seasonal differences ($\chi^2 = 4.5$, $df = 6$, $p = 0.609$) in suicide incidence.

Table 1 and Table 2 show the number of suicides by sex, age, and method. Even when stratified for sex and age, lunar phase was not detected to exert significant influence on the occurrence of either violent or non-violent suicides. Nevertheless, in the group of individuals aged more than 40.2 yrs, both males (10.6%) and females (8.2%) committed fewer suicides using a non-violent method around the absent moon than expected, assuming uniform occurrence of suicide over time. Furthermore, only 11.0% of the violent suicides by females in that age group

TABLE 1 Summary of the Results: The Lunar Phase in Relation to Sex and Chosen Method of Suicides in Subjects Aged Less Than the Median (40.2 yrs)

Method	Absent moon (±48 h)	Interphase (all other days)	Full moon (±48 h)	Total
"Violent"				
n	124	704	133	961
"Suicide rate"[a]	0.948	1.006	1.017	1
Men (% of method)	84 (12.2%)	505 (73.5%)	98 (14.3%)	687 (100%)
Women (% of method)	40 (14.6%)	199 (72.6%)	35 (12.8%)	274 (100%)
"Non-violent"				
n	78	328	53	459
"Suicide rate"	1.249	0.982	0.849	1
Men (% of method)	37 (18.9%)	140 (71.4%)	19 (9.7%)	196 (100%)
Women (% of method)	41 (15.6%)	188 (71.5%)	34 (12.9%)	263 (100%)
Total				
n	202	1032	186	1420
"Suicide rate"	1.046	0.998	0.963	1
Men (% of method)	121 (13.7%)	645 (73.0%)	117 (13.3%)	883 (100%)
Women (% of method)	81 (15.1%)	387 (72.1%)	69 (12.8%)	537 (100%)

[a]"Suicide rate" in relation to the length of each moon phase with each lunar cycle lasting 29.4 days (reciprocal value of the relation of the absolute number (*n*) of suicides in each lunar phase to the absolute number of suicides in each row).

TABLE 2 Summary of the Results: The Lunar Phase in Relation to Sex and Chosen Method of Suicides in Subjects Aged More Than the Median (40.2 yrs)

Method	Absent moon (±48 h)	Interphase (all other days)	Full moon (±48 h)	Total
"Violent"				
n	149	937	165	1251
"Suicide rate"[a]	0.875	1.029	0.969	1
Men (% of method)	108 (12.3%)	655 (74.6%)	115 (13.1%)	878 (100%)
Women (% of method)	41 (11.0%)	282 (75.6%)	50 (13.4%)	373 (100%)
"Non-violent"				
n	36	294	53	383
"Suicide rate"	0.691	1.055	1.017	1
Men (% of method)	20 (10.6%)	143 (76.1%)	25 (13.3%)	188 (100%)
Women (% of method)	16 (8.2%)	151 (77.4%)	28 (14.4%)	195 (100%)
Total				
n	185	1231	218	1634
"Suicide rate"	0.832	1.035	0.981	1
Men	128	798	140	1066
Women	57	433	78	568

[a]"Suicide rate" in relation to the length of each moon phase with each lunar cycle lasting 29.4 days (reciprocal value of the relation of the absolute number (*n*) of suicides in each lunar phase to the absolute number of suicides in each row).

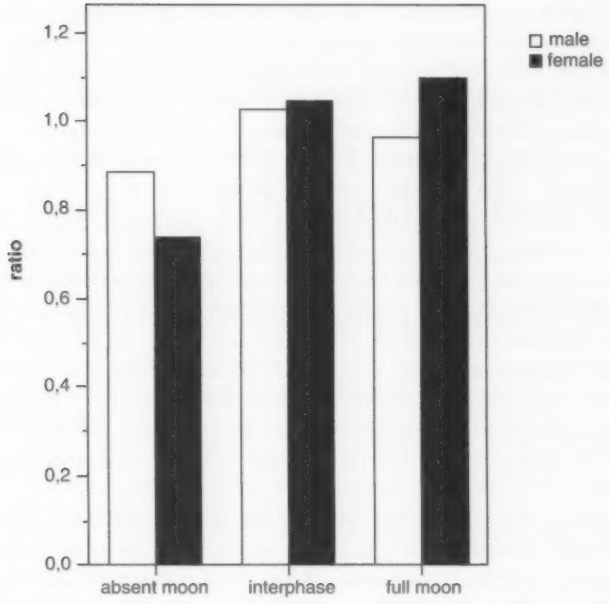

FIGURE 1 Suicide rates at different phases of the moon. Suicide ratio in relation to each phase of the moon: the absent and full moon with an interval of 4 days each centered around the exact date, and the interphase comprised of 21.4 days (reciprocal value of the relation of the absolute number of suicides in each lunar phase to the number of suicides expected assuming the occurrence of suicide is uniform over time).

occured around the absent moon and without a remarkable increase around the time of the full moon. In contrast, 18.9% males and 15.6% females younger than the median age of 40.2 yrs chose a non-violent method to commit suicide around the absent moon; whereas, fewer males (9.7%) committed suicide by a non-violent method within a period of 2 days of the full moon. Although, this was significant in the stratum of men younger than the median age ($\chi^2 = 7.42$, $p = 0.025$), this significance disappeared after we applied a correction for multiple testing.

DISCUSSION

Although this study did not document a significant influence of lunar cycles on the number of suicides by males and females of both age groups, there seemed to be at least a weak effect on the choice of either a violent or a non-violent method around the absent or the full moon phases. As these differences failed to reach statistical significance,

i.e., only a trend in non-violent suicides by men aged less than the median of 40.2 yrs was observed, they might be caused by pure chance.

Despite the fact that several investigations (Garth and Lester, 1978; Gutierrez-Garcia and Tusell, 1997; Maldonado and Kraus, 1991) have repudiated an influence of lunar phases on suicidal behavior, the belief in such a relationship persists (Vance, 1995). Vance (1995) pointed out that psychiatric and mental health personnel (*e.g.*, those who are exposed to intense job-related stress and anxiety) create a kind of association between the full moon and abnormal human behavior, including suicide, to improve their sense of control, as sometimes a superstitious explanation may be preferable to none. This might not be the case in subjects committing suicide, as there are likely to be one or several defined underlying motives for the final act, independent of cosmic events (Kishi and Kathol, 2002), so that the belief in a fictional causal attribution in the moon's influence loses its function. Thus, our findings of a lack of relationship between lunar phase and suicides are not unexpected.

Nonetheless, there are some limitations of our study. As pointed out by Maldonado and Kraus (1991), the time of death is often difficult to determine. Furthermore, it is sometimes difficult to determine whether a given death is the result of an intentional suicide or an unintentional act. As information on the time and manner of death relied on the outcomes of police investigations, the data on the timing as well as the classification of a death as a suicide are dependent on the competence and diligence of the investigators, which is assumed to be high. Furthermore, a larger error might involve the time of death of the act of suicide (Maldonado and Kraus, 1991). If the rate of this error varied by time of day, then this type of error might have caused the observed variation to be greater than the true variation (Copeland et al., 1977). Nevertheless, an advantage of this study lies in the large number of cases, the accuracy of the data, and the completeness of the database of the study population.

CONCLUSIONS

Despite the evidence against a lunar influence on suicide, there continues to be a belief in the moon's effect on human behavior (Gutierrez-Garcia and Tusell, 1997). Individual experience may reinforce those perceptions, which might be related to cognitive biases like selective perceptions or self-fulfilling prophecies. These common beliefs might exert a very small influence on the choice of the method used to commit suicide, of at least some of the individuals at risk for suicide, as well as it might inadvertently impair the judgment of psychiatric or mental health personnel (Vance, 1995).

ACKNOWLEDGMENTS

We thank the members of the Frankonian Police specially Mr. Grill and Mrs. Schönwald from the Police Department Nuremberg/Germany.

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Chronobiology International, 22(6): 1145–1155, (2005)
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ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398130

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SHORT COMMUNICATION

EFFECT OF DIM AND BRIGHT LIGHT EXPOSURE ON SOME IMMUNOLOGICAL PARAMETERS MEASURED UNDER THERMAL NEUTRAL CONDITIONS

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This study assesses the effects of ambient light conditions, under a thermoneutral environment, on selected immunological parameters of 7 healthy young women (aged 19 to 22 yrs). Subjects entered the bioclimatic chamber at 11 : 00 h, controlled at 26°C and 60% relative humidity, a "neutral climate". They lead a well-regulated life in the climatic chamber (*pre*-condition) while exposed to *dim* (200 lux) or, on the next day, *bright* (5000 lux) light between 06 : 00 to 12 : 00 h. Just before the end of each period of light exposure, a blood sample was taken for later immunological assay of white blood cell count (WBC), phagocytosis, interferon-γ (IFN-γ), interleukin-4 (IL-4), CD69 T cells (CD69), $CD4^+CD25^+$ T cells ($CD4^+CD25^+$), and transforming growth factor-β 1 (TGF-β1). The results, when compared with the *pre*-condition, were as follows: 1) CD69 and IFN-γ increased during normal conditions without thermal stress under *dim* light; 2) WBC increased and IL-4 decreased under *bright* light; 3) as shown by the highly significant decrease of TGF-β1, the immune system was activated under *bright* light; 4) phagocytosis tended to increase under *bright* light exposure; 5) CD69 and IFN-γ were significantly higher, and $CD4^+CD25^+$ tended to decrease under *bright* light; 6) phagocytosis tended to be lower and TGF-β1 significantly higher under *dim* light, indicating a decline of immune system function. Taken together, this preliminary single time-point sampling study infers that some parameters are activated (CD69) while others are attenuated (phagocytosis, TGF-β1) according to the environmental light intensity, *dim vs. bright*, in women adhering to a standardized routine in the absence of thermal stress. These findings are discussed

Submitted June 27, 2005, Returned for revision September 16, 2005, Accepted September 22, 2005

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in terms of inhibition of the sympathetic and excitation of the parasympathetic nervous system under the influence of life-style regularity and daytime *bright* light exposure.

Keywords Thermal stress, Regular life, Dim/bright light intensity, Immune activity, Neutrophil, Lymphocyte

INTRODUCTION

The light-dark cycle is an important zeitgeber (Aschoff, 1981; Wever et al., 1983). Daytime bright light exposure has been shown to induce a greater nocturnal fall of core temperature and deeper sleep (Kim and Tokura, 1995, 1998). In contrast, nocturnal bright light exposure inhibits the nocturnal rise of melatonin secretion, which exerts sedative and hypnotic properties (Lewy et al., 1980), and results in a disturbance of deep sleep. Indeed, diurnal bright light increases nocturnal melatonin secretion (Hashimoto et al., 1997; Park and Tokura, 1999) and lowers diurnal core temperature (Aizawa and Tokura, 1998) and diurnal blood pressure (Kim and Tokura, 2000), thereby having a relaxing influence.

The effect of light intensity on the immune system and immunological responses in humans has yet to be studied. The behavior of leukocytes is essential for immunological responses, and among the blood leukocytes, neutrophils, monocytes, and lymphocytes, in particular, play an important role in defense mechanisms. T-cell lymphocytes are an essential part of immune function, making up 70% of the peripheral lymphocytes, and they play an important role in immunologic control. Most importantly, helper T cells secrete various cytokines and enhance immune activity. These leukocytes are under the control of the autonomic nervous system (Suzuki et al., 1997). As the autonomic nervous system is influenced by light (Saito et al., 1996; Nishimura et al., 2003), it is possible the immune system also may be influenced by light. The present experiment was designed to test this hypothesis.

MATERIALS AND METHODS

Subjects and Schedule

Seven healthy female students, aged 19 to 22 yrs, participated. The average times of going to bed nights and awakening mornings were ~23 : 00 and ~07 : 00 h, respectively, during the 1 to 2 wks before study. The subjects were studied either during the follicular or luteal phase (excluding time menses and expected ovulation) of the menstrual cycle. Each participant was exposed to *dim* and *bright* light during the same menstrual phase. None of the subjects used medications, including birth control pills; they were non-smokers and free of infectious or allergic

disorders. The conduct of the research followed the guidelines established by the Journal (Touitou et al., 2004), and all subjects gave informed consent to participate after being informed about the purpose and risks of the study. The study was conducted in mid-summer, between July and August 2004.

Figure 1 shows the experimental schedule. The subjects entered the climatic chamber at 11 : 00 h (*pre*-experimental condition) on the first day. They then relaxed without exercise or napping and wore a soft cotton shirt. The ambient temperature and relative humidity were maintained at 26°C and 60%, respectively, throughout the study. The participants ate standardized meals at 12 : 00, 19 : 00, and 07 : 00 h, and a light snack at 15 : 00 h. A blood sample was collected just before lunch, at 12 : 00 h, each study day. Between 06 : 00 to 12 : 00 h, the room light condition was controlled either at 200 lux (*dim light*) the second day or at 5000 lux (*bright light*) the third day. Lighting was fixed at 100 lux between 12 : 00 and 18 : 00 h and at 50 lux between 18 : 00 and 22 : 00 h every day. The room was kept totally dark during the times allocated to sleep. The subjects were required to gaze at the light periodically, especially during the bright light exposure.

Immunological Assay

Phagocytosis

One hundred μL heparin was added to blood, together with 50 μL fluorescence beads. The mixture was gently stirred for 60 min at 37°C, and

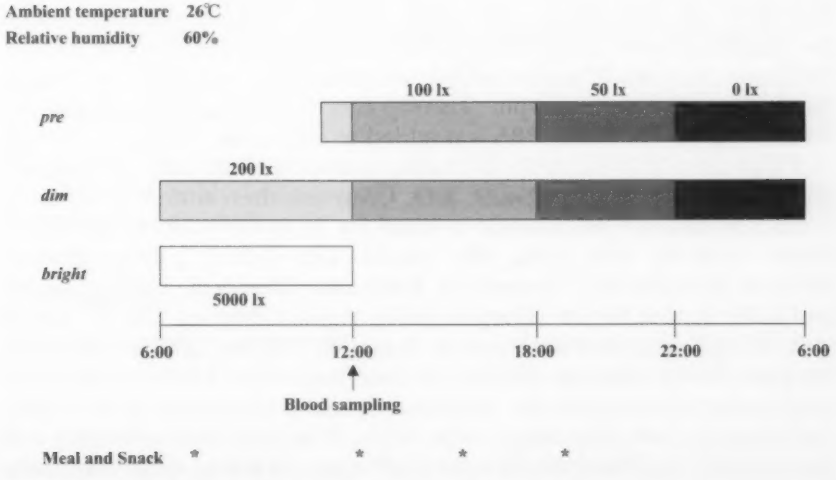

FIGURE 1 Experimental protocol.

then 4 mL phosphate buffer saline (PBS; Medical and Laboratory Co., Nagoya, Japan) and 5 mM ethylene-diamin-tetra-acetic-acid-2Na (EDTA-2Na) were added. The solution was then centrifuged at 4°C for 5 min at 2000 rpm. The top clear layer was removed and the cells resuspended. Four mL lysing solution (Becton Dickinson, San Jose, CA, USA) was added and the mixture stirred gently. The solution was then left for 10 min at room temperature, before centrifuging at 4°C for 5 min at 2000 rpm. Four mL PBS and 5 mM EDTA-2Na were added and the mixture centrifuged for 5 min at 4°C at 2000 rpm. Finally, the phagocytes were analyzed with a flowcytometer (Coulter Elite; Coulter Co., Miami, FL, USA).

Surface Marker Assay

One hundred μL blood and CD69 maker (Immunotech, Marseille, France) were left to stand for 15 to 20 min at room temperature. Two mL lysing solution was added and the mixture kept for 10 min at room temperature. The solution was centrifuged at 4°C for 5 min at 2000 rpm and the cell layer washed with 2 mL PBS and then centrifuged for 5 min at 4°C at 2000 rpm. The cells were analyzed by flow cytometry.

TGF Assay

Two mL lysing solution was added to 100 μL blood and the mixture left to stand for 10 min at room temperature. It was then centrifuged for 5 min at 25°C at 2000 rpm. The top clear layer was removed and the cells resuspended. Two mL of 1% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) were added, and the mixture was left to stand for 10 min at room temperature before centrifuging for 5 min at 25°C at 2000 rpm. Two mL PBS were added and the mixture was then centrifuged for 5 min at 25°C at 2000 rpm. The top clear layer was removed and the cells resuspended. Then, PBS was added to adjust the volume to 100 μL and 5 μL anti-TGF-β1 antibody-Biotin marker added. Ten μL Saponin (Sigma Chemical Co., St. Louis, MO, USA) was then added to adjust to 0.2%. The mixture was allowed to stand for 30 min at room temperature before washing with 2 mL PBS added with 0.05% polyoxyethylene sorbitan monolaurate (Tween-20; Katayama Chemical, Osaka, Japan) and 0.5% Bovine Serum Albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) before centrifugation for 5 min at 25°C at 2000 rpm. Ten μL Streptavidin-PE (Becton Dickinson, San Jose, CA, USA) marker was added and left to stand for 30 min at room temperature. It was then washed with 2 mL PBS added with 0.05% Tween-20 and 0.5% BSA and centrifuged for 5 min at 25°C at 2000 rpm. The top clear layer was removed and the cells resuspended before analysis by flow cytometry.

Intracellular Cytokine Assay

The following reagents were used: RPMI 1640 medium [PMI1640 (Nakarai, Kyoto, Japan), 10% FCS (Sigma Chemical Co., St. Louis, MO)]; Activation medium [RPMI 1640 medium—25 ng/mL PMA, 1 μg/mL Ionomycin (Sigma Chemical Co., St. Louis, MO, USA), 10 μg/mL BrefeldinA (Sigma Chemical Co., St. Louis, MO, USA)]; Control medium RPMI 1640 medium, 10 μg/mL Brefeldin A]; Fluorescein isothiocyanate-labeled monoclonal anti Interferon-γ (Immunotech, Marseille, France); Phycoerythrin-labeled Interleukin-4 (Immunotech, Marseille, France).

Heparinized blood (0.5 mL) was added to each 0.5 mL medium and the mixture warmed for 4 h at 37°C. After warming, 3 mL PBS were added to a 200 μL sample of the mixture. The sample was centrifuged for 5 min at 4°C at 2000 rpm. The top clear layer was removed and the cells resuspended. Then, 10 μL CD4, 8-PC5 was added and the mixture left to stand in the dark for 15 min at room temperature. Next, 4 mL lysing solution was added, the mixture left for 10 min at room temperature, and then centrifuged for 5 min at 4°C at 2000 rpm. Four mL 5% formaldehyde-PBS was added and the mixture centrifuged for 5 min at 4°C at 2500 rpm. The top clear layer was removed and the cells resuspended. Five hundred μL permeabilizing solution (Becton Dickinson, San Jose, CA, USA) was added and the mixture was placed in the dark for 10 min. Three mL 0.5% BSA-PBS was added and the mixture was centrifuged for 5 min at 4°C at 2500 rpm. The supernatant had 10 μL anti IFN-FITC and 10 μL anti IL-4-PE added and then placed in the dark for 15 min at room temperature. Three mL PBS was added and the mixture centrifuged for 5 min at 4°C at 2500 rpm. The top clear layer was removed and the cells resuspended and analyzed by flow cytometry.

Statistical Analysis

All data are shown as mean ± SE. Differences between mean values were assessed by Student's paired t -test, with statistical significance documented when $p < 0.05$.

RESULTS

CD69, IFN-γ, and TGF-β1 increased significantly (Figures 2 and 3) following the sudden transition from the *pre*-experimental to the *dim* light condition, with the subjects following a standardized routine in a thermal neutral environment. In contrast, phagocytosis tended to be lower as a result of this change (Figure 2). WBC, CD69, and IFN-γ were significantly higher and IL-4 significantly lower in the *bright* light condition

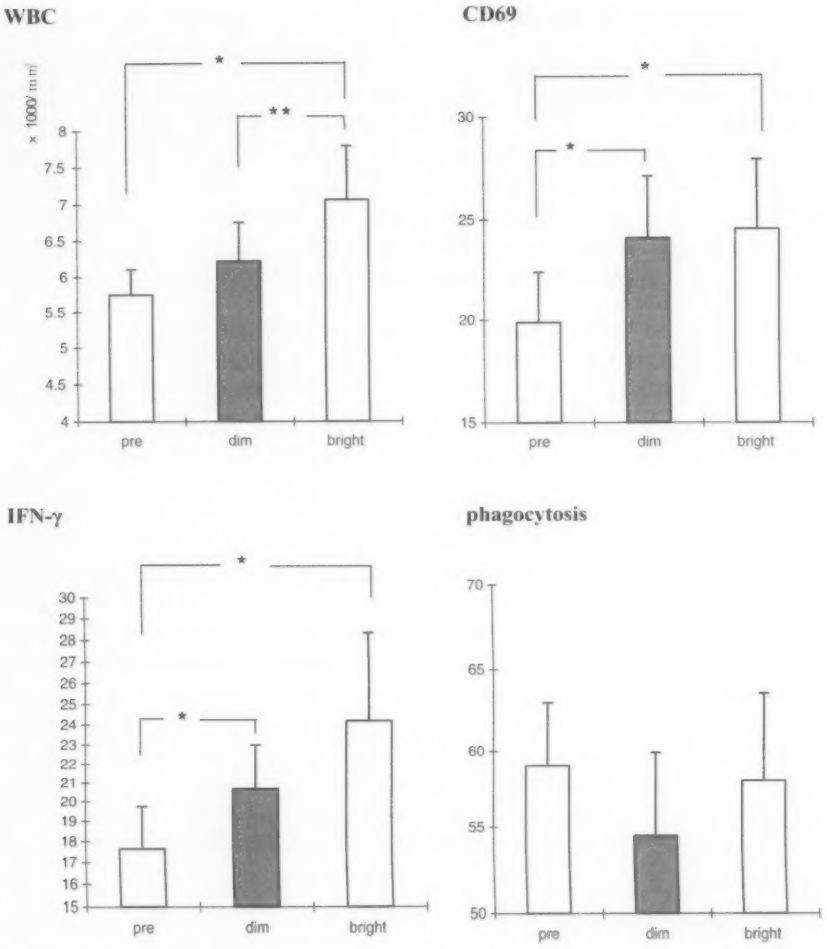

FIGURE 2 WBC, CD69, IFN-γ, and phagocytosis under the influence of pre, dim, and bright conditions. $^{*}p < 0.05$, $^{**}p < 0.01$, Mean ± SE, $n = 7$.

than in the *pre*-condition (Figures 2 and 3). Furthermore, WBC increased significantly in the *bright* light condition compared to the *dim* light condition (Figure 2), and IL-4 and TGF-β1 decreased significantly in the *bright* compared to the *dim* light condition (Figure 3). Phagocytosis tended to recover the value of the *pre*-condition under the transition from the *dim* to *bright* light condition (Figure 2). $CD4^{+}CD25^{+}$ decreased gradually from the *pre*-condition through the *dim* to *bright* light condition (Figure 3).

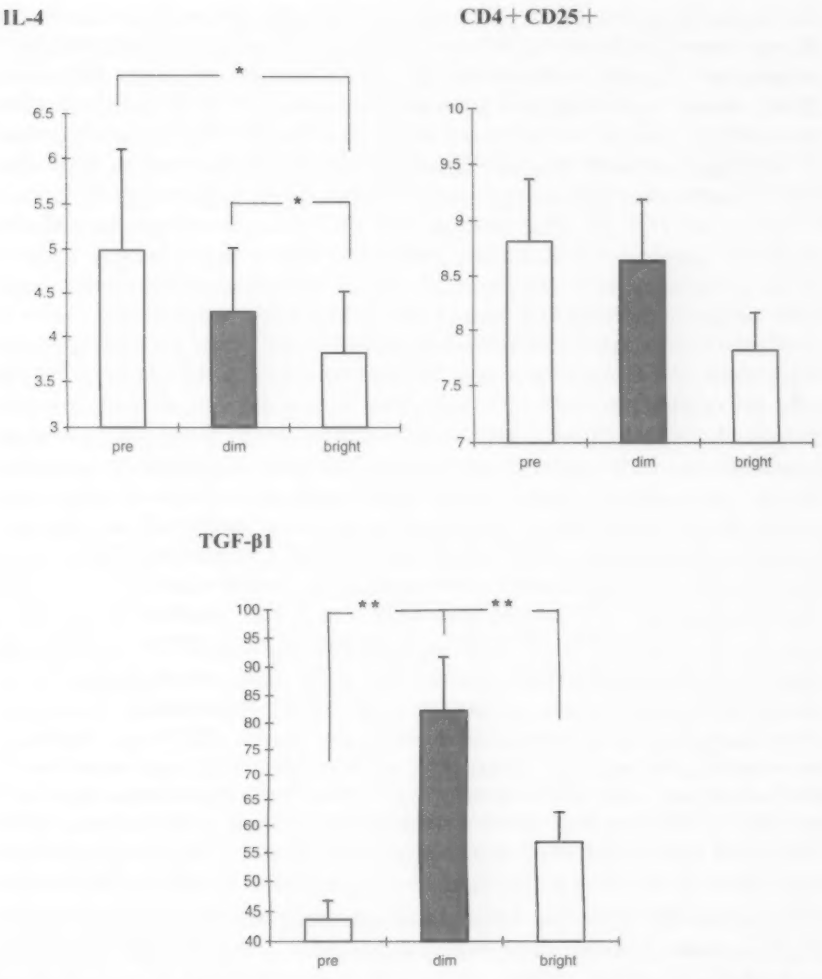

FIGURE 3 IL-4, $CD4^+CD25^+$, and TGF-β1 under the influence of pre, dim, and bright condtions. $^*p < 0.05$, $^{**}p < 0.01$, Mean $\pm$ SE, $n = 7$.

DISCUSSION

Under non-stressing ambient thermal neutral conditions, CD69T cells (Hara et al., 1986), an indicator of lymphocyte activity, and IFN-γ increased significantly. When diurnal *bright* light exposure was introduced, in addition to reduced thermal stress, WBC increased and IL-4 decreased significantly. Also, IFN-γ and CD69T cells were greater in the *bright* than *pre*-light condition. Phagocytosis tended to recover activity under

the *bright* light condition. TGF-β1 was remarkably reduced with the change from the *dim* to *bright* light condition. None of the immunological parameters showed reduced activity after diurnal *bright* light exposure. Thus, many immunological parameters increased their activity under the study condition of no thermal stress and diurnal *bright* light exposure.

TGF-β1 reduced immunological activity by its increase in level (see below) under *dim* light, suggesting that the *bright* light is significant for reduction of TGF-β1. The finding that TGF-β1, one of cytokines that is generally present in all tissues and cells and which strongly inhibits immunological activity (Blobe et al., 2000), fell significantly under *bright* light; suggests the immune system was enhanced by *bright* light.

CD69T cells and IFN-γ increased under *dim* light. However, under *bright* light, WBC increased and IFN-γ improved. $CD4^+$ helper T (Th) cells are classified into Th1 and Th2 cell types, depending on the nature of the cytokines secreted (Mosmann et al., 1986). The finding that the anti-inflammatory cytokine IL-4 was suppressed and that IFN-γ was induced under *bright* light indicates a bias of Th2 over Th1 cell type production. This appears to be of biological significance. Reiner and Locksley (1995) found as Th1 cells produced IFN-γ, they killed *Leishmania major* and mice became infection-resistant (Th1 cell type superiority). If, in contrast, IFN-γ was not produced, but IL-4 and IL-10 were (Th2 cell type superiority), mice died by accelerating activity of *Leishmania major* within the cells; also, macrophages were inactivated under these cytokines and IFN-γ production inhibited. Thus, immunological responses were active under Th1 type superiority, resulting in infection resistance, while under Th2 type superiority, immunological responses were suppressed, resulting in infection susceptibility. Our present results suggest the risk of infection was likely decreased since Th1 type superiority was induced by the *bright* light condition. It should be noted that $CD4^+CD25^+$ T cells tended to be reduced under the *bright* light condition, because they suppress other T-cell activity and proliferation (Takahashi et al., 1998; Thornton and Shevach, 1998, 2000). Thus, it seems beneficial in terms of health maintenance to avoid heat stress and be exposed to diurnal bright light.

What physiological mechanisms might be responsible for these findings? Diurnal bright, rather than dim, light exposure for several hours induces higher urinary melatonin secretion (Aizawa and Tokura, 1999), lower tympanic temperature (Aizawa and Tokura, 1998), and lower blood pressure (Kim and Tokura, 2000) during daytime activity. These physiological responses are accompanied by relaxation of the body and, probably, suppression of the sympathetic and enhancement of the parasympathetic nervous systems. Leucocytes are affected by the autonomic nervous system, and lymphocytes have receptors for

acetylcholine (Suzuki et al., 1997), suggesting that lymphocytes are controlled by the parasympathetic nervous system. Leucocytes may play an important role in body defense mechanisms, and the autonomic nervous system is known to be influenced by light (Saito et al., 1996; Nishimura et al., 2003). Taking both these factors into consideration, enhanced activity of the parasympathetic nervous system triggered by diurnal bright light might stimulate certain components of the immune system.

The subjects were quiet and relaxed without thermal stress during the entire experimental period. The ambient conditions were kept comfortable, with room temperature of 26°C and 60% relative humidity, and the subjects also were under a strictly controlled daily routine in terms of the hours of waking and retiring, time of meals, and so on. It seems that such strictly controlled schedules, without heat stress, also could account, at least in part, for enhanced activity of immunological parameters. Some immunological parameters are suppressed under severe thermal stress in humans (Frohlich et al., 2004) and chickens (Mashaly et al., 2004), and subjective sleep quality is improved with a more regular lifestyle (Monk et al., 1997, 2003). This combination of reduced thermal stress and improved sleep quality strengthens defense mechanisms, which is thought to reduce the risk of infectious and malignant diseases (Bryant et al., 2004).

The limitations of this study are the relatively small number of study subjects, the systematic exposure of subjects to the (*dim* and *bright*) light conditions, and the single circadian time of blood sampling. Despite these limitations, the study findings infer the enhancement of immunological parameters on the third day of the protocol did not result from habituation of the subjects to environmental conditions of the bioclimatic chamber, but rather the influence of the 6 h daytime bright light exposure. Since rapid relaxation of the body (within 6 h under the influence of bright light exposure in the morning) has been previously reported (Aizawa and Tokura, 1998, 1999; Kim and Tokura, 2000), we conclude that morning exposure to 6 h bright light, together with a strictly regulated lifestyle in thermal neutral conditions, can enhance several immunological parameters.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Jim Waterhouse in Liverpool, UK for helpful suggestions and comments in reading our manuscript. This research was supported in part by financial assistance from Sekisui House Co., Ltd.

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Chronobiology International, 22(6): 1157–1335, (2005)
Copyright © 2005 Taylor & Francis LLC
ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500398155

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ABSTRACTS

THE FIRST INTERNATIONAL CONGRESS OF APPLIED CHRONOBIOLOGY AND CHRONOMEDICINE (ICACC)

The 1st International Congress of Applied Chronobiology and Chronomedicine was held in Kemer, Antalya, Turkey, June 1–5, 2005. A total of 154 scientists and students from 28 countries participated in the scientific program that consisted of 10 symposia, 5 plenary lectures, and 10 oral/poster, free-communication/discussion sessions.

The conference featured plenary lectures by Alain Reinberg (Euchronism, Allochronism, and Dyschronism in Clinical Chronobiology), E. Haus (Application of Basic Chronobiology to Chronomedicine), B. Bruguerolle (General Chronopharmacology), M.J. Sole (Day/Night Rhythms as Determinants of Cardiovascular Health and Disease), and E.J.W. Van Someren (Improving Sleep by Thermal Manipulation).

The ten symposia addressed the topics of "Circadian Clocks in Cancer Processes" (presentations by C. Focan, F. Levi, E. Filipski, and P. Innominato); "New Developments in Cancer Chronotherapy" (presentations by G. Bjarnason, C. Focan, S. Giachetti, X.M. Li, and C. Garufi); "Chronobiology of Nutrition, Appetite Control, and Obesity" (presentations by E. Haus, S.P. Kalra, K.T. Borer, L.M. Morgan, and Y. Touitou); "Chronobiology and Chronotherapy of Hypertension" (presentations by M.H. Smolensky, F. Portaluppi, and R. Hermida); "Light Therapy for Psychiatric Disorders: Which Patients Will Benefit?" (presentations by A. Wirz-Justice, R. Lam, K. Martiny, and F. Benedetti); "Gender Issues in Chronomedicine" and "Chronobiologic Optimization of Medical Treatments in Women" (presentations by R. Correa, E. Haus, B. Bruguerolle, M.H. Smolensky, F. Portaluppi, R. Hermida, F. Levi, W. Hrushesky, and R. Lam); "Chronobiology of Sleep and Fatigue Disorders" (presentations by Y. Dagan, A. Wirz-Justice, S. Ancoli-Israel, and A. Szeinberg); "Shift Work and Health Consequences" (presentations by F.M. Fischer, A. Lowden, C.R.C. Moreno, and M.L.N. Pires); "Melatonin: Hormone and Drug" (presentations by Y. Touitou, C. Ekmekcioglu, C. Bartsch, S. Tordjmann, and B. Claustrat); "Circadian Rhythms in Dementia" (presentations by S. Ancoli-Israel, D.G. Harper, E.J.W. Van Someren, J. Martin, and P. Zee).

The meeting abstracts of this congress, the 1st international meeting dedicated entirely to chronomedicine, were edited by the senior editor of journal for publication here. Great effort has been extended to avert errors and to preserve the meaning and intent of the authors.

Hakan Zengil, PhD
Professor of Pharmacology
President-Organizing Committee
1st International Congress of Applied Chronobiology and Chronomedicine

Michael Smolensky, PhD
Co-editor-in-Chief, Chronobiology International

PLENARY LECTURE

EUCHRONISM, ALLOCHRONISM, AND DYSCHRONISM IN CLINICAL CHRONOBIOLOGY

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Objective: New findings related to internal desynchronization (ID) in man and its inter-individual variability lead to the reconsideration of some basic definitions. In the same subject, during longitudinal studies ($n \geq 8$ days), the circadian period of a set of variables can differ from one another and from 24 h, even in the presence of natural zeitgebers. This was documented for *e.g.*, activity/rest, oral temperature, heart rate, grip strength of both hands, and cognitive performances (Reinberg et al., 1984, 1994, 1997, 2003; Motohashi, 1990; Ashkenazi et al., 1997; Shub et al., 1997; Chandrawanshi and Pati, 2000). Several methods, including power spectra analyses, helped to demonstrate that ID can be observed in many circumstances: shift work, placebo effect on healthy subjects, work in the high Arctic Summer, sport training, etc.

Results: Four types of symptoms characterize the intolerance to shift work: persistent fatigue, sleep alterations, behavioral changes, permanent use of sleeping pills (which are of no help) (Andlauer, et al., 1979; Reinberg, et al., 1988; 1992). Good tolerance (symptom free subjects) to shift schedules was found in 56% of workers and the poor tolerance in 44%, based on a series of prospective studies involving more than 140 shift workers. ID was found in all non-tolerant shift workers, but also in tolerant ones. In a

group of 40 healthy day workers, ID was found in 35%, with gender-related differences. In addition, emphasis is given to the fact that many diseases, *e.g.*, nocturnal asthma, chronic pain, persisting anxiety and stress, fibroma, and prostate adenoma, involve chronic deprivation of sleep at night and constitute conditions that mimic the presentation of non-tolerant shift workers with ID.

Conclusions: ID appears to be a rather common phenomenon. In some individuals, ID causes no clinical symptoms and the new concept of "allochronism" [allo = different] is proposed to designate a variant of human temporal organization with no pathologic implication. The term "dyschronism" [dys = abnormal] is then restricted to ID associated with a set of symptoms similar to those of non-tolerant shift workers. The term "euchronism" [eu = normal] may be used when circadian parameters (τ, Ø, A, and M) of documented variables are within the confidence limits of pertinent reference values.

Keywords Circadian period, Internal desynchronization, Persistent fatigue, Euchronism, Allochronism, Dyschronism

APPLICATION OF BASIC CHRONOBIOLOGY TO CHRONOMEDICINE

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The circadian (about 24 h) rhythms are directed and maintained by cellular oscillators, which create a transcriptional-translational feedback system and are found in most if not all, mammalian cells. The system is kept in step with its periodic environmental surrounding by photic stimulation from the retina through a non-vision related pathway acting upon the suprachiasmatic nuclei (SCN) which serve as pacemaker and central master clock directing the circadian time organization. The central oscillator relates to the peripheral oscillators and target tissues through humoral (neuropeptides and hormones) and neural (direct or multisynaptic) connections. Peripheral oscillators, *e.g.*, in liver, heart, vascular walls, etc. are also amenable to other entraining stimuli, *e.g.*, the time of food uptake. Circadian periodicity affects about 2 to 10% of mammalian genes. Phase and extent of circadian clock-dependent gene expressions vary in different organs and tissues. Circadian clock genes are involved in regulating cell proliferation and apoptosis through control of cell

cycle related genes and tumor suppressor genes. In rodents, defects in the clock gene Per 2 lead to a tumor diathesis with increased sensitivity to environmental carcinogens, *e.g.*, ionizing radiation and to high mortality. Defects in cryptochrome, another part of the clock system are linked to defects in the cell cycle and in tissue repair and regeneration. In human subjects, a polymorphism in the clock-gene Per 3 is found to be associated with an increased risk to develop breast cancer.

Oscillators have been found in all parts of the cardiovascular system with the circadian oscillators regulating the production and activity of plasminogen activator-inhibitor, which in part determines the circadian rhythm in the incidence of thromboembolic phenomena, being of special interest. Changes in Per 2 and Per 3 also are related to the advanced and delayed sleep phase syndrome and to behavioral morning or evening preference. Changes in Per 1 and Per 2 clock genes have been found in rodent models to be associated with drug and alcohol addiction. There is tentative evidence for a similar role in human alcoholism. In sudden phase shifts, as they occur in shift work or transmeridian travel, there occurs an internal desynchronization on a molecular basis within the oscillators and a transient loss of control of the central pacemaker over the peripheral oscillators with internal and external desynchronization, which may lead to symptoms (shift work intolerance and/or jet lag symptoms). Frequent and often repeated phase shift may be associated with cardiovascular complications and perhaps even with some forms of cancer.

Knowledge of the molecular mechanisms of the human circadian time structure may open the way for rhythm adjustment and preventive and/or therapeutic intervention through the development of chronobiotic agents.

Keywords Chronomedicine, Oscillators, Clock genes, Desynchronization

SYMPOSIUM I–CIRCADIAN CLOCKS IN CANCER PROCESSES

CIRCADIAN TIMING SYSTEM AND CELL CYCLE INTERACTIONS

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Background: The circadian timing system coordinates daily activities at organismic, cellular and molecular levels, while the cell cycle/apoptosis

and DNA repair system controls cell proliferation and tissue homeostasis. Quality of life can be viewed as resulting from proper adjustment of endogenous rhythms to environmental cycles at the organism level. Clearing of genetically altered cells and adequate replacement of cell loss are key processes for a normal lifespan.

Objective: To identify the relationships between the circadian timing system and the cell cycle. Both rhythmic systems display complex interactions that could represent useful novel targets for cancer therapeutics.

Methods: Experiments were conducted in $B6D2F_1$ or C3H/He healthy or tumor-bearing mice kept in LD12:12. Bone marrow, liver, or tumor cells were sampled every 3 to 6 h to determine cell cycle phase distribution with flow cytometry, protein expression with western blot, mRNA expressions of clock genes or cell cycle genes with RNase protection or qPCR. The circadian timing system was altered through suprachiasmatic nuclei (SCN) ablation or simulated chronic jet lag exposure.

Results: The proportions of S and G2/M phase cells respectively peaked near the middle and end of darkness in the bone marrow of $B6D2F_1$ mice kept in LD12:12, while G1 cells peaked near midlight. Similar G1, S, and G2/M rhythms were found in mice with suppressed rhythms in rest-activity and body temperature as a result of SCN ablation. In these mice, the count of nucleated cells was significantly increased as compared with sham-operated mice. Thus, the SCN control cell proliferation threshold; yet, circadian cell cycle gating involved different mechanisms. The occurrence of a sustained circadian rhythm in the *in vitro* response of cultured bone marrow cells to GM-Colony Stimulating Factor further supported the autonomy of the circadian rhythms in cell proliferation from the SCN. Although clock genes *Per2* and *Bmal1* were expressed in mouse bone marrow, their circadian mRNA expression patterns displayed atypical amplitudes and phase relation. However, anti-apoptosis protein BCL-2 and pro-apoptosis BAX varied 3- to 5-fold along the 24 h time scale in the same bone marrow extracts. In mouse MA13/C mammary cancer, a significant circadian gating was found for G2/M transition but not for G1/S or BCL2. In subsequent mouse studies, clock genes *Rev-erbα*, *Per2*, and *Bmal1* displayed an altered phase relation in rapidly proliferating Glasgow osteosarcoma; yet, *c-Myc* (a key gene for G1/S transition) apoptosis regulation, and carcinogenesis tended to be expressed rhythmically. Ongoing studies reveal that anticancer drugs which interfere with the cell cycle can alter host circadian physiology and molecular clocks.

Conclusions: Peculiar features characterized the molecular regulation of healthy or malignant proliferating tissues along the 24 h time scale. The understanding of the complex interactions between both rhythmic systems at physiological and molecular levels will provide new therapeutics against

malignant and other diseases in which cell cycle, apoptosis, or DNA repair are altered.

Keywords Cell cycle, Cancer therapeutics, Mice, Clock genes, Apoptosis, DNA repair

CIRCADIAN DISRUPTION IN EXPERIMENTAL CANCER PROCESSES

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Objectives: Circadian rhythm alterations independently predicted for poor survival outcome in cancer patients. Frequent transmeridian flights or predominant work at night increase cancer risk, possibly as a result of disrupted coordination of circadian clocks. We investigated the consequence of severe circadian dysfunction caused by suprachiasmatic nuclei (SCN) destruction or experimental jet-lag on tumor growth in B6D2F1 mice and the prevention of circadian dysfunction with feeding schedules.

Methods: Three series of experiments were performed: 1) The SCN of mice were destroyed by electrocoagulation and then, 3 wks later, they were inoculated s.c. with a transplantable Glasgow osteosarcoma (GOS) or pancreatic adenocarcinoma (PO3), 2) Mice were randomly divided in 2 groups, one was kept in LD 12:12 and the other phase advanced by 8 h by advancing the light-dark cycle every 2 days to produce chronic jet-lag (CJL). All mice were inoculated with GOS 10 days after jet-lag onset, 3) GOS-bearing mice were submitted to CJL or to CJL and meal timing (MT) to prevent circadian rhythm alterations. In all experiments locomotor activity and temperature were recorded by telemetry. Time series were analyzed by spectral and Cosinor analyses. Mice survival was checked daily and tumor growth was measured 3 times a week. Mice with tumor weight reaching 2 g were sacrificed for ethical reasons and considered as dead from tumor progression on this date. In all experiments plasma corticosterone concentrations and lymphocyte counts were assessed every 4 h over a 24 h span, and in jet-lag experiments the mRNA expression of clock genes (*Per2*, *Reverb-a* and *Bmal1*) and genes involved in cell cycle progression (*c-Myc* and *p53*) was determined in liver and tumor with RT-PCR.

Results: The 24 h rest-activity cycle was ablated and serum corticosterone and lymphocyte count markedly altered in lesioned mice as compared to sham-operated animals. The growth rate of either tumor was 2 to 3 times faster in lesioned than in sham mice. In GOS-bearing mice, mean tumor weight (±SEM) on day 12 (before the death of the first animal) was

1443 ± 203 mg *vs.* 490 ± 93 mg in lesioned and in sham mice, respectively (*t*-test; $p = 0.002$). In P03-bearing mice mean tumor weight on day 22 was 1447 ± 342 mg in lesioned and 749 ± 136 mg in sham mice (*t*-test; $p = 0.05$). CJL markedly altered the rest-activity, temperature and corticosterone rhythms in all the mice. Tumor grew faster in these animals as compared to those kept in LD 12:12 (p from ANOVA < 0.001). On day 11, mean tumor weight was 1330 ± 151 mg in jet-lagged mice and 647 ± 56 mg in controls (*t*-test; $p = 0.001$). Significant circadian rhythmicity in per2, bmal1 and reverbα mRNAs expression was observed in the liver and tumor of control mice; whereas, no obvious rhythm was detected in p53 or c-Myc. CJL shifted clock gene rhythms in the liver and ablated them completely in the tumor. It also caused down regulation of p53 expression ($p = 0.002$), up regulation of c-Myc and induced a 12-fold circadian variation in c-Myc mRNA ($p = 0.03$) in the liver of healthy mice; whereas, complex interactions were found in tumor-bearing animals. Subjecting the mice to the timed meal regimen restored circadian rhythmicity in clock gene expression in jet-lagged animals and retarded malignant progression.

Conclusions: These results suggest that environmental conditions such as altered light-dark or feeding schedules modify circadian clock coordination at the physiological and molecular levels and play a significant role in malignant progression. This finding warrants the development of therapeutic strategies aimed at treating or preventing circadian clock dysfunctions along the course of cancer processes.

Keywords Jet-lag, Molecular clock, Feeding schedules

RELATIONSHIP BETWEEN CIRCADIAN TIME STRUCTURE OF HOST AND TUMORAL OUTCOME (HUMAN DATA)

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The importance of circadian rhythmic signaling by EGFR (as well as ligands EGF and TGF-α) within the paraventricular suprachiasmatic nuclear efferent zones in the control of some aspects of the circadian behavior (*i.e.*, temperature and motor activity) has been documented recently. Perturbations in animals of these central rhythmic signals either by local infusion of TGF-α or EGF, destruction of SCN and ventricular zones (SPZ), or chronic jet-lag will promote tumor growth. Also, the appearance of cancer clones in the liver after nitrosamine exposure may be observed

after loss of the circadian corticosterone control and may be promoted by continuous light exposure. In humans, the risk of breast or colon cancers is increased in flight attendants.

In cancer patients also alterations of circadian rhythms (*i.e.*, tumor markers, cortisol, melatonin, protein, WBC, etc) are frequently observed. An accurate evaluation of the quality of the circadian structure of the host may be easily achieved either by cortisol (16 *vs.* 8 h values), melatonin (serum or saliva sampling), or by wrist actimetry for a duration of 3 to 5 days. Studies have been performed in colorectal (and also lung cancers).

Precisely, in colorectal cancer (CRC), 2 studies are of great interest:

1. A retrospective analysis on 192 patients with metastatic CRC (C. Mormont & F. Lévi, Villejuif, France), among whom 61% had received previous treatment, established the importance of the quality of the circadian rest-activity rhythm as an independent variable of predicting survival.
2. The same evaluation was performed more recently in the 05963 EORTC study on 141 chemotherapy naïve CRC patients. Here also in multivariate analysis, the circadian rhythmicity power was related to survival.

Interestingly, both studies have also shown a clear relationship between the 24 h rest-activity rhythm and quality of life (*i.e.*, fatigue, insomnia, among others), or depression items.

The role of cytokines produced by the tumors (e.g., TGF-α, IL6,) and their implications in the behavior, quality of life, cortisol and 24 h rest-activity pattern via the SCN/SPZ circuit are currently under investigation (P. Innominato, Chieti, I; T. Rich, Charlottesville, USA).

Keywords Host rhythmicity, Actimetry, Cancer prognosis

TUMOR CYTOKINES AND CIRCADIAN RHYTHMS OF HOST: RELEVANCE FOR QUALITY OF LIFE (QOL)

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Background: Pts suffering from previously treated metastatic colorectal cancer (MCC) can display altered circadian rhythms in rest/activity. These alterations are correlated with poor quality of life. In experimental models, the rest/activity rhythm is suppressed by Tumor Growth Factor-α (TGF-α), and hPer1 expression is induced by Interleukin-6 (IL-6).

Objectives: To confirm the relation between the rest/activity rhythm and QoL in chemotherapy-naïve MCC pts (study I). To investigate the effects of TGF-α and IL-6, two tumor-associated cytokines, on the 24 h rest/activity rhythm of cancer pts (study II). To evaluate the effects of EGFR-Tyrosine Kinase inhibitor gefitinib (ZD1839) on the 24 h rest/activity rhythm, quality of life, and serum cytokine levels in pts with non-small cell lung cancer (NSCLC) (study III).

Methods: The rest/activity rhythm was assessed with a wrist piezo-electric accelerometer for 3 days (Ambulatory Monitoring Inc., USA). Two previously validated robust circadian parameters were considered, r24 and I < O (Mormont MC, Clin. Cancer Res. 2000). The EORTC quality of life questionnaire (QLQ-C30) was completed by each pt. In study I, 94 MCC pts were assessed before the first course of chemotherapy, and 71 had a second evaluation after 4 courses (EORTC 05963 trial). In study II, 80 MCC pts with poor (r24 < 0.35) or good (r24 > 0.47) rest/activity circadian rhythm had TGF-α and IL-6 determined in serum obtained at 08:00 h (ELISA) and serum cortisol measured at 08:00 and 16:00 h (RIA). In study III, 7 of 40 planned NSCLC pts received gefitinib (250 mg po/die, International Expanded Access Programme with ZD1839) for more than 14 days. The rest/activity rhythm was assessed before the start of treatment and every 2 weeks on therapy.

Results: In study I, r24 and I < O were significantly correlated with global quality of life, physical, role, social functioning, fatigue, appetite loss, pain, and insomnia ($|r| \geq 0.30$; $p < 0.001$), both at baseline and after 4 courses of chemotherapy. Treatment-associated changes will be reported. In study II, poor rest/activity rhythm was associated with large increase in median serum TGF-α ($\times$4.6-fold; $p = 0.002$) and IL-6 ($\times$1.5-fold; $p = 0.006$). Fatigued pts had significantly higher serum TGF-α ($\times$5.3-fold; $p = 0.006$), while anorexic pts had higher IL-6 ($\times$1.6-fold; $p = 0.01$), TGF-α ($\times$4.4-fold; $p = 0.01$), and mean serum cortisol ($\times$1.3-fold; $p = 0.01$). No correlation was found between the cortisol rhythm and quality of life or symptoms. Study III is ongoing, and partial results will be presented.

Conclusions: We demonstrate for the first time in a multicenter international setting the strong correlation between the subjective quality of life and the circadian rest/activity rhythm, thus an objective and quantitative estimate of QoL. We show the association between tumor-associated cytokines and disrupted circadian rest/activity rhythm of the host and symptom clusters in cancer pts. We further study the relevance of an

interventional approach to target this mechanism for improving the QoL of cancer pts.

Keywords Rest-activity circadian rhythm, Quality of life, Cytokines/ Growth factors

PLENARY LECTURE

GENERAL PRINCIPLES IN CHRONOPHARMACOLOGY

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Among many factors of variability, the response of the organism to a drug or a toxic agent depends on the time of its administration. Chronopharmacology studies the influence of the moment of administration of a drug (hour, month, or year) on its response according to the temporal structure of the organism receiving it. Chronopharmacology also studies drug-induced alterations of biological rhythms. Three aspects of chronopharmacology may be distinguished:

Chronotoxicology: the acute and/or chronic toxicity of a drug varies according to the time of its administration.

Chronopharmacodynamics: The time of administration of a drug may determine a different response from a qualitative or a quantitative point of view. The qualitative response of an organism to a drug (i.e., inhibition or induction, increase or decrease of its effect) as well as the quantitative response (duration or intensity of the action) varies with time of its administration.

Chronopharmacokinetics: The time of administration is a factor of nonlinearity of drug kinetics. Time dependent-changes in absorption, distribution, metabolism, and excretion have been reported for many drugs in animals and in humans. Chronokinetic data may partly explain chronopharmacological phenomena. Moreover, knowledge of the influence of the time of administration on the kinetics of a drug may have implications for its prescription by modulating the distribution of the total daily dose along the 24 h scale.

Models and concepts have been developed in chronopharmacological studies, such as transgenic animals in cardiovascular chronopharmacological studies, for instance, and *in vitro* studies in cultured cells. New tools such as implantable radiotelemetry systems allowing undisturbed measurement of chronobiological parameters in conscious and unrestrained animals, microdialysis probe implantations permitting continuous analysis by

tissular approach, and pumps allowing a continuous constant or sinusoidal administration of drugs have been introduced in chronopharmacology.

Clinical chronopharmacology and chronotherapy proceeds from chronobiological and chronopharmacological data: the choice of the moment of drug administration may optimize therapeutic effects by reducing its side effects and/or enhancing its efficacy. Several chronotherapeutic studies have been reported in different areas, such as oncology, allergy, pneumology, and rheumatology.

The principles and objectives for a chronopharmacological study are discussed: when, how, and where to conduct such a study and the place of chronopharmacological studies during the different stages of drug development.

Keywords Chronopharmacology, Chronopharmacokinetics, Chronopharmacodynamics, Chronotherapy

SYMPOSIUM II-NEW DEVELOPMENTS IN CANCER CHRONOTHERAPY

PRECLINICAL CHRONOPHARMACOLOGY OF NEW DRUGS

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Objectives: The therapeutic index of most anticancer drugs may be improved by adequate selection of the circadian time of their administration. The relevance of circadian dosing-time dependency was investigated as a prerequisite toward the development of chronotherapeutic schedules of gemcitabine (GEM), an S-phase specific cytotoxic agent, and L-Alanosine (SDX-102), an inhibitor of purine synthesis.

Methods: B6D2F_1 mice were synchronized to 12 h dark:12 h light cycle prior to receiving single or multiple drug doses for toxicity studies or multiple doses for antitumor efficacy studies. Chronoefficacy was studied in mice with 1) Glasgow osteosarcoma (GOS) treated with GEM ± cisplatin (CDDP) 1' or 4 h later or 2) P388 leukemia receiving SDX-102. The drugs were administered at one of six circadian times (3, 7, 11, 15, 19 or 23 h after light onset–HALO). The effects of both drugs on the circadian rhythms in body temperature and activity were further investigated in unrestrained mice implanted with a radiotelemetry transmitter.

Results: GEM caused least body weight loss and least neutropenia after injection at 11 *vs.* 23 HALO, whether it was given alone or with CDDP

(ANOVA; $p \leq 0.003$). GEM-CDDP tolerability was improved by dosing GEM at 11 HALO and CDDP at 15 HALO ($p < 0.001$). The administration of this schedule to GOS-bearing mice increased median survival 3-fold as compared to treatments where both drugs were given simultaneously at 11 or 23 HALO (Log rank $p = 0.02$). The circadian amplitudes of body temperature and activity were markedly damped following GEM at 23 HALO, but were not modified after GEM at 11 HALO (ANOVA; $p < 0.05$).

SDX-102 dosing at 19 HALO reduced lethality 3- or 5-fold after single or multiple doses as compared to the worst time (p from $\chi^2 < 0.01$). Neutropenia, lymphopenia, and bone marrow hemorrhagic lesions were significantly less in mice dosed at 19 *vs.* 7 HALO. The survival of P388 mice was prolonged by dosing at 7 or 23 HALO (Log rank $p = 0.03$). SDX-102 dosing at 7 HALO transiently ablated the 24 h patterns in body temperature and activity.

Conclusions: Tolerability and efficacy were simultaneously improved by the delivery of GEM in the late rest span and SDX-102 in the late activity span, in relation with circadian rhythms in cell cycle phase distribution and metabolic pathways in purine synthesis. Furthermore, alterations of circadian outputs by GEM or SDX-102 delivery at the most toxic time suggest that the tumor control by the circadian timing system may be alleviated by toxic processes. The optimal delivery schedules in humans would correspond to an administration of GEM upon awakening, CDDP near mid-activity, and SDX-102 near the end of the activity span.

Keywords Circadian rhythm, Gemcitabine, Alanosine, Chronotherapeutics

LESSONS FROM THE EORTC CHRONOTHERAPY STUDIES PERFORMED IN COLORECTAL CANCER: NEW AVENUES

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The EORTC Chronotherapy Group (ECG) is the most relevant cooperative group studying chronobiology and chronotherapy in cancer medicine. Centers belonging to the groups come from Europe and Canada, with 20 active centers and 20 probational centers. The most important tumor type investigated by the ECG is advanced colorectal cancer (ACC), a disease where important progress has been made in the last 15 years. From a single agent active in this disease in the early '90s, 5-fluorouracil (5-FU)

modulated by leucovorin (FA), different drugs were introduced, such as oxaliplatin (L-OHP) and irinotecan (CPT-11). Moreover, this scenario is rapidly changing due to the recent application of "target therapies" represented by monoclonal antibodies, such as cetuximab and bevacizumab, directed against the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF). In these years the ECG developed a strategy based on three types of trials: a) "proof of principle" trials: we compared a time scheduled delivery of 5-FUFAL-OHP (FFL) with a flat infusion of the same three drugs, showing a reduction (5-fold for mucositis and 2-fold for sensitive neuropathy) in toxicity, an increase in response rate from 30 to 50%, and in time to progression without any increase in overall survival; b) "trials comparing chronotherapy with standard therapies": EORTC trial 05963 compared chronomodulated FFL 4-10, a 4-day schedule, with the FOLFOX2 regimen, a 2-day schedule. The aim of this study was to increase survival by chronomodulation in 554 ACC patients. The study showed no difference in the primary end-point but a significant difference in survival according to gender. Males treated by chronomodulation survived significantly better than males treated with FOLFOX2, while it was opposite for females. Two different patterns of toxicity were also found. Diarrhea was prevalent in the chrono arm *vs.* neutropenia in the standard arm. The question if this gender effect is due to chronomodulation or to schedule needs to be further investigated. The third type of trial is c) the "time finding study, in the current EORTC 05011 where two new concepts are investigated: the addition of CPT-11 to our previous FFL combination and the verification of the best peak timing of CPT-11, randomizing patients to 6 different peak timing for CPT-11. Accrual of this trial is very near goal. The future implication of intensifying treatment with this triplet combination in patients with liver-only metastases represents one possible direction of this research. In this general context, other supplemental important information is being derived from our studies: the relevance of the rest-activity circadian rhythm (RAR) as an independent prognostic factor for ACC patient survival was prospectively confirmed for the first time in a multicenter setting in EORTC 05963 trial together with a strong correlation with items related to patient Quality of Life (ASCO '05). RAR was also found to be linked to alterations in cytokine production, such as IL-6, TNF, and TGF-α, which are partly responsible for patient symptoms (Clin. Cancer Res. 2005). New emerging information regarding clock genes, the correlations of RAR and growth factors, and the interference of drug delivery on the circadian system, constitute the next steps of our investigations in advanced colorectal cancer.

Keywords Colorectal cancer, Cancer chronotherapy, Oral mucositis, Neuropathy, Gender differences, Circadian sleep-wake rhythm

A RANDOMIZED PROSPECTIVE PHASE-III STUDY COMPARING THE ACUTE ORAL MUCOSITIS OF MORNING *VS.* AFTERNOON RADIOTHERAPY (RT) IN PATIENTS (PTS) WITH SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (SCCHN): NCIC-CTG HN.3

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Background: Acute oral mucositis is dose-limiting in SCCHN pts receiving RT. This study was based on our demonstration of a circadian rhythm in human oral mucosa proliferation with the majority of cells in G1-phase in the morning, S-phase in early afternoon, G2-phase in late afternoon, and M-phase at night (*Am. J. Pathol.* 154:613) with an associated rhythm in clock-gene expression (Am. J. Pathol. 158:1793). Clock-genes control the circadian rhythm in cell proliferation (Cell 111:41; Science 302:255). RT does not change the timing of the circadian peak for mitotic index in mouse cornea (Radiat. Res. 89:65). Since cells in G1 are more radioresistant than cells in G2/M, it was hypothesized that morning RT would lead to less acute mucositis.

Methods: 216 pts were randomized to morning (08:00–10:00 h) *vs.* afternoon (16:00–18:00 h) RT in 12 centers. Pts with SCCHN receiving radical or postoperative RT encompassing a visible area of oral and/or oropharyngeal mucosa were eligible. Pts were stratified by RT dose (50 Gy/25 fractions (fr) *vs.* 60 Gy/30 fr *vs.* 60 Gy/25 fr *vs.* 66 Gy/33 fr *vs.* 70 Gy 35 fr), smoking status during RT, and center. Oral mucositis was scored using RTOG criteria and a validated scoring system (Cancer 85:2103). Standardized supportive care was allowed. The primary endpoint was the incidence of at least one RTOG grade 3/4 oral mucositis.

Results: Of 205 evaluable pts, 52.9% *vs.* 62.4% developed Grade 3/4 mucositis with AM *vs.* PM RT (adjusting for smoking status and RT dose; $p = 0.18$). After 5 months post-therapy, there was more weight loss in the PM RT group. In an exploratory subgroup analysis on 111 pts with target RT doses > 60 Gy (66 Gy/33 and 70 Gy/35), there was a 66% relative reduction in grade 3/4 mucositis (44.6% *vs.* 67.3%; $p = 0.022$) with AM RT. In this subgroup, the time to development of grade 3/4 mucositis was longer with AM RT (median > 7.9 weeks for AM RT *vs.* median 5.6 weeks for PM RT; $p = 0.033$). In 53 pts who smoked during RT there was less grade 3/4 mucositis (42.9% *vs.* 76%; $p = 0.025$) with AM RT. Smoking is known to increase RT-induced mucositis.

Conclusions: This proof of principle trial did not show the expected reduction in mucositis when all pts/all doses where included in the analysis. The apparent reduction in mucositis with AM RT in a large (111 pts.) subgroup of pts with target RT dose > 60 Gy suggests that RT timing should be

studied further in cancer sites where gastrointestinal toxicity is dose-limiting. The circadian rhythm in normal tissue proliferation should be considered in future radiobiology studies.

Keywords Squamous cell carcinoma, Radiotherapy, Oral mucositis, Cancer chronotherapy

A MULTICENTER RANDOMIZED TRIAL TO DETERMINE THE OPTIMAL CIRCADIAN TIME OF VINORELBINE ADMINISTRATION COMBINED WITH CHRONOMODULATED INFUSION OF 5-FU IN PREVIOUSLY TREATED PATIENTS WITH METASTATIC BREAST CANCER. (EORTC 05971 STUDY)

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Objectives: To define the least toxic time of Vinorelbine administration given in combination with chronomodulated (22:00-10:00 h) infusion of 5-FU. Primary endpoint: incidence of grade 3 to 4 neutropenia over the first 3 courses. Secondary endpoints: other toxicities and dose intensities.

Treatment scheme: Vinorelbine, 30 mg/m^2/d D1 and D6 and 5-fluorouracile 850 mg/m^2 from D2 to D5, repeated every 21 days. After the inclusion of 46 pts the dosage of Vinorelbine was reduced to 25 mg/m^2/d due to toxicity. Vinorelbine dosing time was randomized every 3 h starting at 00.00 h. Randomization was stratified according to previous chemotherapy (2nd *vs.* 3rd line) and institution. Fixed dose reductions were allowed according to toxicity.

Statistical design: The least toxic time was estimated using a logistic regression model assuming a sinusoidal distribution over time (*i.e.*, over the 8 different arms) of the toxicity rate observed in each arm. The associated 90% confidence limits (CL) were determined by the bootstrap method. Parametric methods for the estimation of CL were used to check the consistency of results (Baron et al., Controlled Clin. Trials 2001; 22: 50S–60S).

Inclusion criteria: Women presenting with histologically proven metastatic breast cancer, having given their written informed consent according to ICH/EU GCP, national/local regulations; WHO performance status $\leq$2; prior administration of at least 1 line of chemotherapy

for metastatic disease, adjuvant or neoadjuvant chemotherapy being considered as first line if the interval between the adjuvant or the neoadjuvant and the palliative chemotherapy less than one year; minimum of 4 weeks between the last sequence of chemotherapy or radiotherapy and inclusion in the study.

Exclusion criteria: Prior high-dose chemotherapy; prior radiation therapy except for primary tumor; axillary or mammary chain treatment; concurrent anti-tumor or steroid therapy; biology contraindicating chemotherapy; patent cardiac insufficiency or ischemic disease; patent cerebral metastases and bowel obstruction; patent bronchoconstriction outside pulmonary lymphangitis; any serious chronic disease; or pregnancy or nursing.

Results: From 1999 until 2004, 90 pts, with metastatic breast cancer were recruited. Seven pts were excluded from analysis due to cerebral metastasis (1), prior bone radiotherapy (2), insufficient delay between the last treatment and inclusion in study, (2) and no treatment after randomization (2). From the 83 remaining pts, 40 were assigned to the 30 mg/m^2 regimen while 43 received the 25 mg/m^2 one. Vinorelbine was administered at 00:00 h in 12, 03:00 h in 11, 06:00 h in 10, 09:00 h in10, 12:00 h in 9, 15:00 h in 10, 18:00 h in 10, and 21:00 h in 11. Treatment violations were encountered leading to under treatment of 30 pts (pump technical problem in 24, dosage mistake in 2, and miscellaneous reasons in 4), or over treatment of 9, essentially because of dose reduction as per protocol. Some 67 pts (81%) received 3 cycles, 7 (8%) only 2 cycles, and 9 (11%) only 1 cycle of chemotherapy. A total of 224 cycles could be analyzed (40, 33, 27 first, second, and third cycles for the Vinorelbine 30 mg/m^2 regimen pts and 43, 41, 40 first, second, and third cycles for the Vinorelbine 25 mg/m^2 regimen pts.

Dose intensity: Vinorelbine and 5FU relative dose intensities were globally 79.4% and 78.2% in the Vinorelbine 30 mg/m^2 group, it while was 88.1% and 87.4% in the Vinorelbine 25 mg/m^2 group.

Toxicity: Over the 3 cycles, toxicity by cycle was encountered. Grade 3 and 4 leucopenia in 47% and 29%, grade 3 and 4 neutropenia in 12% and 77%, grade 3 febrile neutropenia in 34%, and grade 2 thrombopenia and anemia—4% each. The main other grade 3 and 4 toxicities were cardiovascular (2% and 2%), lethargy (4%), diarrhea (4% and 1%), vomiting (1% and 1%), stomatitis (12%), constipation (2% and 4%), other gastrointestinal (5% and 1%), infection (4%), sensory (4%), pulmonary (2% and 3%), and alopecia (7%).

Reason off study: 81% of the pts underwent the full protocol; 12% were terminated for toxicity; 5% for progressive disease; 1% for pt refusal; and 1% for intercurrent death unrelated to treatment.

Time finding study: The logistic regression model determined the optimal time of administration of Vinorelbine for leucopenia grade 3 to 4

to be ~17:15 h (Table), with the range between 14:00 and 20:00 h. No optimal time could be determined for the other toxicities.

Time 90% CI	30 mg/m² Dose	25 mg/m² Dose	Both doses	Stratification by dose
Leucopenia	17:36 h	17:03 h	17:17 h	17:15 h
Gr 3–4	07:33-21:59 h	13:21-20:42 h	13:58-20:2 h	14:17-20:10
Neutropenia	20:02 h	24:00 h	20:57 h	20:57 h
Gr 3–4	02:05-23:44	00:00-24:00 h	01:09-23:28 h	01:15-23:29
Neutropenia	19:51 h	07:33 h	07:40 h	07:40 h
Gr 4	02:43-22:50 h	02:56-11:35 h	01:25-22:23 h	01:32-22:00 h
Febrile	03:12 h	03:12 h	21:39 h	03:23 h
neutropenia	01:27-22:53 h	00:50-23:10 h	00:48-23:13 h	00:59-23:08 h
Gastrointestinal	09:55 h	14:14 h	10:42 h	10:27 h
Gr 3–4	06:56-12:03 h	04:22-20:30 h	07:15-14:50 h	06:58-14:15 h

Conclusions: Chronomodulation of Vinorelbine in humans demonstrated an optimal time of administration at 17:00 h to reduce the incidence of leucopenia grade 3 to 4. This time of administration almost corresponds to the least toxic time and to the most efficient time of administration in the animal model (19 HALO). Other toxicities are not modified by the time of administration of Vinorelbine. This is the first time that randomized clinical studies in pts (and logistic regression with bootstrap method) have been found to reinforce chronobiological animal pre-clinical data.

Keywords Vinorelbine, 5FU, chronotolerance, Chronotoxicology, Cancer chemotherapy

Supported by grants: Pierre Fabre Oncology, Ligue Bourguignonne contre le cancer.

ORAL PRESENTATION SESSION A
DISTORTED CIRCADIAN FUNCTION IN PATIENTS WITH ADVANCED NON-SMALL CELL LUNG CANCER

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Objectives: Animal experiments show that distorted circadian function accelerates tumor progression and shortens survival, while restoring normal circadian function enhances the survival benefits of chemotherapy.

We report on the circadian function of pts with advanced non-small-cell lung cancer. There are no data in the literature on the circadian function of pts with this common cancer.

Methods: Thirty-three pts with a histologic diagnosis of Stage III or IV non-small cell lung cancer were recruited at Cancer Treatment Centers of America and Dorn Veterans Administration Medical Center into a randomized clinical trial investigating the clinical benefits of melatonin. After signing a consent form, participants wore a wrist actigraph to record the number of accelerations/min for 4 continuous days. The actigraph data were translated into sleep/activity parameters through the Act Millennium and Action W2 software and Chronolab v.2.

Results: The cosinor parameters—the 24 h amplitude and circadian quotient—revealed increasing disruption in circadian function with increasing ECOG physical status scores. At all ECOG physical status scores, pts were experiencing significantly distorted circadian function. Interestingly, pt self-report of sleep quality (Pittsburgh Sleep Quality Index) was indistinguishable from insomniacs. In addition, Quality-of-life data form the EORTC-QLQ-C30 and QLI demonstrated a significantly diminished physical function and dissatisfaction with quality of life.

Conclusions: Advanced non-small cell lung cancer pts have a significantly distorted circadian rhythm. Therefore, follow-up data on these pts' circadian function will provide information on the clinical benefits of exogenous melatonin.

Keywords Circadian Rhythms, Non-small-cell lung cancer, Melatonin, Quality of life, Actigraphy

CONTINUOUS LIGHT INCREASES CANCER GROWTH RATE IN CLOCK GENE REPLETE, BUT NOT PER2 MUTANT, MICE

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Objective: The regular alternation of light and darkness of the natural environment resets the host central circadian clock, which daily maintains the circadian control of essential physiological functions in normal and tumor-bearing hosts. Pulsed or continuous nocturnal light exposure disrupts the delicate circadian organization. Circadian disruption increases cancer risk. mPer2 mutations are associated with spontaneous neoplasia

and hPer3 polymorphism with increased breast cancer risk. mPer2 mutants do not respond normally to constant bright light, failing to promptly lose its free-running circadian rhythm, and showing abnormal phase response to timed light pulses.

Methods: We measured tumor growth rate and its circadian organization in female C3Heb/FeJ and C57BL/6 mice (wild-type and mPer mutants), bearing, respectively, transplanted syngeneic breast and colon cancer during a span of LD12:12, followed by a span of constant (LL) 300 Lx full spectrum light. We measured circadian wheel running activity rhythms in the C3H mice under both conditions, and we recorded the estrous cycle stage, daily, in all mice. We also measured mitotic index and selected core clock gene protein levels in tumors obtained from mice living under LD and LL conditions.

Results: Constant light, immediately, obliterates host circadian locomotor activity rhythms and reduces overall activity levels among clock gene replete C3H mice. In clock gene replete mice of each strain, within 24 h of constant light exposure, tumor growth rate doubles. Constant light is associated with a 50% increase in tumor cell mitotic index ($p = 0.02$), four-fold decrease in tumor cell mPer1 protein level ($p = 0.03$), and two-fold decrease in tumor cell mBmal-1 protein ($p = 0.03$). This acceleration of tumor growth is not explained by concurrent changes in body weight or estrous cycling patterns (all mice continue to cycle). In colon cancer-bearing per2 mutant mice, however, constant light exposure does not accelerate tumor growth.

Conclusion: We conclude that, constant light increases cancer cell proliferation and tumor growth rate through a pathway that requires normal mPer2 function.

Keywords Ambient light cycle, Clock gene replete mice, Per2 mutant mice, Tumor growth rate

NEO-ADJUVANT AND ADJUVANT CHRONOTHERAPY FOR THE MANAGEMENT OF ESOPHAGEAL AND GASTRIC CANCERS

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Introduction: A fully ambulatory chronotherapy protocol [including 5-fluorouracil (5FU) and folinic acid (FOL) (respectively, 700 mg/m^2/day and 300 mg/m^2/day times 5 infused from 22:00 to 10:00 h with a peak of delivery at 04:00 h) and carboplatin (50 → 60 mg/m^2/day times 5;

infusion from 10:00 to 22:00 h with peak delivery at 16:00 h); courses repeated every 3 weeks] was developed for advanced esophageal and gastric cancer.

Esophageal cancer: 52 pts (median age: 63 yrs, range: 46 to 80 yrs; sex: M/F: 32/20) suffering from advanced esophagus cancer, either of the squamous cell (35) or adenocarcinoma (17) type were included in the therapeutic program. 25 cases had distant metastases. The chronotherapy was well tolerated (grades 3 to 4, percentage pts: mucitis 19%, diarrhea 8%, WBC 19%, granulocytes 50%, platelets 35%), although requiring drug-dose/delivery adjustment in 63% to 69% of pts, especially in those receiving concomitant radiotherapy. The response rate was 79%, with 33% complete responses (CR); the level of response was not influenced by the presence of metastases; responses were seen in 97% of pts receiving concomitant radiotherapy. Unfortunately, the loss of 8 pts after curative surgery limited the one-year survival to 40%.

Gastric cancer: The same protocol was proposed to 56 pts (median age: 61 yrs, range: 32 to 81 yrs; sex M/F: 41/15) suffering from gastric carcinoma, as adjuvant postoperative or as palliative treatment. 39 pts were considered as stage IV disease. The toxicity was comparable to that observed in esophagus cancers (grades 3 to 4, percentage pts: WBC 18%, granulocytes 45%, platelets 18%, nausea-vomiting 2%, mucitis 11%, and diarrhea 7%) with dose adjustments needed by half the pts. 61% of pts showed a palliative response. The time to disease progression was, respectively, 64.4 months in adjuvant *vs.* 5.2 months in palliative pts; the median survival was 13 m in palliative-treated pts and was not attained in adjuvant-treated pts (72% at 5 + yrs).

Conclusions: An interesting therapeutic index with limited toxicity, high response rates, and good survivals (at least in stomach cancers) was observed. The ambulatory treatment increased pt quality of life. Confirmation of the results is warranted through phase III comparative trials *vs.* other chemotherapy programs (*e.g.*, Cunningham, DCF, etc) and newer drug development (e.g., vinorelbine or gemcitabine for esophageal, oxaliplatin, docetaxel, and irinotecan for gastric cancers).

Keywords Chronotherapy, Ambulatory medicine, Esophagus cancer, Gastric cancer

PROLONGED MONITORING OF REST-ACTIVITY RHYTHM FOR ASSESSING CHEMOTHERAPY EFFECTS ON CIRCADIAN SYSTEM

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Rationale: The circadian rhythm in rest/activity, monitored for 3 days, was significantly correlated with quality of life and fatigue, and it was an independent prognostic of survival in cancer patients [Mormont et al., Clin. Canc. Res., 2000]. Anticancer treatments can also alter circadian physiology and molecular clocks in mice [Ohdo et al., Nature Med., 2001; Li et al., Chronobiol. Int., 2002].

Objectives: To assess the effects of anticancer treatments on the circadian rhythm in rest-activity of cancer patients.

Methods: The rest-activity circadian rhythm was monitored for 1 month in 10 pts (5 men, 5 women, 34 to 83 yrs old), with advanced cancer of the ovary (2 pts), colon or rectum (6 pts), or lung (2 pts). The wrist actigraph Mini-Motionlogger (Ambulatory Monitoring Inc., USA) was worn on the non-dominant wrist for 1 week before chemotherapy delivery, during it, and for ≥ 2 weeks after chemotherapy. Quality of life was evaluated with weekly EORTC QLQ-C30 questionnaires. The Karolinska sleep log questionnaire was completed at the end of each recording session. The circadian rhythm in activity was estimated by the: 1) autocorrelation coefficient, r24, a measure of the regularity of the activity pattern over 24 h, 2) dichotomy index, $I < O$, the percentage of activity counts in bed (I) below the median activity counts out of bed (O).

Results: 4 pts with ovarian or lung cancer received gemcitabine or docetaxel-based chemotherapy (chronomodulated for 3 pts); 6 pts with metastatic colorectal cancer received chronomodulated 5-fluorouracil–leucovorin-oxaliplatin and/or irinotecan and/or cetuximab; 8 pts had received extensive prior chemotherapy. Before the course of chemotherapy, median r24 and $I < O$ values were 0.43 (range: 0.18 to 0.60) and 98.2% (range: 92 to 100), respectively. During the week when chemotherapy was given, r24 increased by >30% in 2 pts and decreased by >30% in 6 pts. The dichotomy index ($I < O$) was reduced by >8% in 5 pts. Over the subsequent 2 weeks, r24 and $I < O$ remained lower than pretreatment values in 5 and 2 pts, respectively.

Conclusion: Chemotherapy delivery can produce sustained changes in the rest-activity circadian rhythm of cancer pts. The relationship between these alterations and other aspects of the circadian physiology or molecular clocks awaits further exploration. Early information on changes in the rest-activity 24 h pattern may guide subsequent tailored optimization of treatment dose and timed delivery schedule.

Keywords Cancer, Chronotherapy, Rest-activity, Circadian rhythm, Toxicity

Supported by RNTS Project AILISA n 03 B 656, Università "G. D'Annunzio" Chieti, ARTBC Internationale Hôp. P. Brousse, Villejuif

RELATION BETWEEN ANTITUMOR EFFICACY OF CYCLIN-DEPENDENT CYCLIN KINASE INHIBITOR SELICICLIB AND DRUG EFFECTS ON CLOCK AND CELL CYCLE GENES

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Background: Seliciclib (SEL) is a cyclin-dependent kinase inhibitor with best antitumor activity following dosing during the rest span of mice (Zeitgeber time, ZT3 and ZT11) and poorest efficacy at ZT19. Interaction between the circadian timing system and cell cycle could explain these results.

Objective: To study the relationship between chronoefficacy of seliciclib, clock genes, and cell cycle gene expression patterns.

Methods: Glasgow osteosarcoma was inoculated to 132 male $B6D2F_1$ mice synchronized to 12 h L and 12 h darkness SEL (300 mg/kg/day) or vehicle were administered 9 d later via daily oral gavage for 5 d—at ZT 3, 11, or 19—to 96 drug-treated and 36 control animals. Tumors were sampled at ZT 5, 11, 17, and 23 to determine the mRNA circadian expression of mPer2, mBmal1, mc-Myc, mWee1, and m36B4, as a reference invariant gene, using quantitative two-step PCR.

Results: We report preliminary results on 72 tumor samples. No significant circadian rhythm in clock genes or Wee1 expression was found in tumor of control mice, while c-Myc was rhythmic with an acrophase at ZT 9 (p from cosinor = 0.04). SEL dosing at ZT3 or ZT11 induced a significant rhythm in Per2 expression with an acrophase of ZT9 or ZT5, respectively ($p < 0.0001$ and $p = 0.02$). SEL at either dosing time also resulted in significant variations in c-Myc and Wee1 expression, with peaks differing by 12 h. Conversely, following SEL at ZT19, the Per2 rhythm peaked at ZT20 ($p < 0.0001$) and c-Myc and Wee1 displayed synchronous variations peaking at ZT17. These results were validated with 2-way ANOVA (p for interaction < 0.05).

Conclusions: The molecular clock was altered in untreated tumor animals, in agreement with previous data. Optimal efficacy of SEL during rest may result from the control of cell cycle coordination by the molecular

clock. Adequate targeting of therapeutic agents to the clock gene machinery may improve cancer control.

Keywords CDKi, Circadian rhythm, Cell cycle, Cancer, Chronotherapeutics

Supported by Cyclacel Ltd, Dundee, UK, ARTBC Internationale, Hôp. Paul Brousse, Villejuif, France; Università "G. D'Annunzio," Chieti, Italy

ORAL PRESENTATION SESSION B

THE CONTROL OF THE DAILY RHYTHM IN PLASMA GLUCOSE CONCENTRATIONS BY THE BIOLOGICAL CLOCK INVOLVES THE AUTONOMIC INNERVATION OF THE LIVER

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Objectives: Daily peak plasma glucose concentrations are attained shortly before awakening. We investigated the neuroanatomical pathways used by the biological clocks, the suprachiasmatic nuclei (SCN), located in the hypothalamic to generate this anticipatory rise in plasma glucose concentrations.

Methods: Aided by microdialysis techniques, SCN transmitter (ant)agonists were administered into the paraventricular nucleus (PVN) of the hypothalamus, *i.e.*, a target area of the SCN, of awake and freely moving rats. At the same time, blood samples were taken to analyze plasma glucose, insulin, glucagons, and corticosterone concentrations.

Results: We show that stimulation of NMDA-receptors, or blockade of GABA-receptors in the PVN, of conscious rats causes pronounced increase in plasma glucose concentrations. An important part of these inhibitory PVN inputs proved to be derived from the SCN. Moreover, the hyperglycemic effect of NMDA and the GABA-antagonist showed pronounced diurnal variation. Combination of GABA-receptor blockade in the PVN with selective removal of either the sympathetic or parasympathetic branch of the hepatic autonomic innervation showed that the hyperglycemia produced by PVN stimulation was mainly due to an activation of the sympathetic input to the liver. Food deprivation did not attenuate the hyperglycemic effect of the GABA-antagonist induced activation of the PVN, despite the almost complete disappearance of hepatic glycogen.

Conclusions: We propose that the daily rise in plasma glucose concentrations is caused by a SCN-mediated withdrawal of GABAergic inputs to the sympathetic pre-autonomic neurons in the PVN, resulting in an

increased hepatic glucose production by increased gluconeogenesis. Since pts with type-2 diabetes often show inappropriately high hepatic glucose production, these results clearly demonstrate that malfunctioning of the SCN-PVN-axis may contribute to the development of type-2 diabetes. Presently, we are investigating how PVN stimulation affects the rhythmic expression of liver genes involved in glucose metabolism and whether it also affects the expression of clock genes in the liver.

Keywords Glucose, GABA, Hypothalamus, Sympathetic, Liver, PVN, SCN

BLUE AND RED BIOPTRON LIGHT INFLUENCES MOTOR UNITS ACTIVITY AND SKIN TEMPERATURE

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Objectives: The aim of this study was to verify if different spectra of visible incoherent polarized light emitted by Bioptron (Bioptron Light Therapy System is a medical device; BIOPTRON AG, Switzerland) influence temperature and activity of muscle motor units in healthy volunteers.

Methods: Tests were performed on 10 healthy females aged 23 to 24 yrs in the early morning hours (mainly at 08:00 h.) in January. Five were exposed to Bioptron light with a blue filter, while the others were exposed to it with a red filter. The wavelength of the Bioptron light ranges from 480 to 3,400 nm, and its intensity is about 300 lux. Continuous measurements of temperature were recorded from the surface of the skin over the m. extensor carpii muscle, while electromyographic (EMG) and electroneurongraphic (ENG, M-wave) examinations were performed before and after light exposure subsequently from the masseter, extensor carpii, and abductor digitii minimi muscles, and ulnar nerves on both sides. All recordings were compared to the control group that received no exposure ($n = 20$).

Results: After red light exposure, skin temperature increased about 1.5°C. A significant EMG amplitude increase was found in most of the study volunteers ($n = 12$; $p = 0.05$). There were no changes in the frequencies of action potentials during voluntary movements, and there

was only moderate increase in the amplitudes of the resting potentials when muscles were relaxed. Blue light exposure did not evoke similar changes, except a moderate increase in amplitude of the EMG recordings during muscle contraction. Surprisingly, amplitudes of resting potentials slightly decreased ($n = 10$; $p = 0.01$). Also, the temperature rose only ~0.5°C. All phenomena were most frequently seen subsequently in the extensor carpii, masseter, and abductor digitii minimi muscles. Neither spectra of light influenced efferent fiber transmission in the ulnar nerves.

Conclusions: Our study confirms that Bioptron light improves the activity of muscle motor units; however, greater changes are visible after exposure to red than blue light.

Keywords Visible incoherent polarized light, Motor units, Red spectrum, Blue spectrum, Skin temperature

THE BRIGHT LIGHT AFFECTS GENERAL MUSCULAR ACTIVITY (EMG) IN HEALTHY SUBJECTS

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Objectives: In our previous study (*J. Physiol. Pharmacol.*, 2002; 53: 1-33), we shoved bright light (3000 lux) significantly affects motor unit activity in forearm muscle of healthy subjects tested in the winter season in the early morning hours. The present experiments were undertaken to establish if the responses to bright light exposure are different in the winter and autumn seasons (as the bright light of 3000 lux is applied commonly in the treatment of winter depression) and if these responses are related to the time of day of exposure and/or are sex-dependent.

Methods: The studies were performed on 10 healthy females and 10 males 18 to 40 yrs of age, both in winter (December, average environmental temperature, ~4°C) and in autumn (September, average environmental temperature ~18°C). Tested subjects were exposed to bright light emitted by fluorescent lamp Foto Vita for 60 min, 3 times per day (at 08:00, 14;00, and 20:00 h). Electromyographic (EMG) activity of the motor units in the extensor carpii muscles, electroneurographic studies (ENG) of the efferent fibers transmission in radial nerves (M wave), and skin surface temperature over the extensor carpii muscles were measured before and

after each light exposure. All results were compared to parameters obtained in a group of 50 persons not exposed to bright light.

Results: The EMG studies and skin temperature measurements showed a significant increase in the tested parameters after light exposure, unrelated to the time of the day ($p = 0.05$). These changes were more accentuated in women than men ($p = 0.01$). However, no significant changes in ENG in radial nerves (M wave) were found.

Conclusions: Our study confirms an influence of the artificial bright light on electrophysiological parameters of motor units of the tested skeletal muscle, particularly in females. However, the bright light effects on general muscular activity (EMG) seem to be unrelated to season (at least winter *vs.* autumn) and time of day.

Keywords Bright light, EMG (Electromyographic Activity of Motor Units), ENG (Electroneurographic Studies of the Efferent Fibers Transmission)

DAILY VARIATION IN PLASMA LIDOCAINE LEVELS: IMPLICATIONS FOR NEUROPROTECTION?

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Objectives: Daily variation in the duration of action of the local anesthetic lidocaine is well documented. The independent role of lidocaine as a neuroprotectant is currently under investigation. Here we report that time of day affects circulating plasma lidocaine levels in pts receiving a 12 h intravenous infusion of lidocaine during and after cardiac surgery.

Methods: 46 subjects received a 1 mg/kg bolus of lidocaine followed by a 2 h I.V. infusion of 2 mg/min and a 10 h infusion of 1 mg/min. Plasma titers were obtained 2 h (sample 1) and 10 h (sample 2) after the infusion start. The relationship between plasma levels, body weight, and time of day were investigated.

Results: Plasma lidocaine levels were poorly correlated with body weight ($r^2 = 0.04$; $p > 0.5$). However, the mean concentration of sample 1 was 6.6 μg/mL in morning cases (10:40 h) and 8.3 μg/mL in afternoon (15:20 h) cases ($p = 0.028$). The mean concentrations of sample 2 were 5.6 μg/mL (21:00 h) and 6.4 μg/mL ($p > 0.05$), respectively.

Conclusions: A significant time of day difference in plasma lidocaine levels exists during constant infusion, with higher levels in the afternoon. This may reflect reduced metabolism of the drug in the afternoon. We are currently collecting data with increased temporal resolution. Confirmation of a temporal variation in plasma lidocaine levels may help explain disparities in the literature on the efficacy of lidocaine as a neuroprotectant. Examination of the relationship between time of day and lidocaine's neuroprotective effect will be conducted on trial completion.

Keywords Lidocaine, Circadian rhythm, Anesthesia, Cardiac, Neuroprotection

CIRCADIAN EFFECTIVENESS OF SOLAR RADIATION

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Objectives: The effectiveness of solar radiation at the Earth's surface to modulate circadian rhythms by suppression of melatonin concentration depends on different factors, such as solar elevation angle, atmospheric transparency, adaptation of the eyes, and spectral transparency of their lenses.

Methods: To determine the threshold angles of solar elevation to induce sufficient melatonin suppression, measurements of the spectral solar irradiance received by both a horizontal and a vertical area were performed at solar elevations between -6.5° and $+84^\circ$ under the conditions of both clear sky and completely covered sky (dark nimbostratus clouds). Data of effective irradiance for melatonin suppression were derived using the action spectra of melatonin suppression according to Thapan and coworkers (2001), Brainard and colleagues (2001), and Gall and Lapuente (2002) with respect to age-dependent changes of lens transparency.

Results: The threshold irradiance to cause sufficient melatonin suppression is exceeded between sunrise and $\sim$30 min before sunset for clear and cloudless skies. If the sky is covered by nimbostratus clouds, the threshold elevation angle increases up to $\sim 13^\circ$ for clear lenses and to $\sim 20^\circ$ for more turbid lenses.

Conclusions: Depending on latitude and season, these limits decrease the suitable time of day to stimulate melatonin suppression by solar radiation and show the need to use suitable artificial light sources to prevent as well as to treat disorders due to disturbances of the circadian rhythm of melatonin suppression.

Keywords Melatonin suppression, Solar irradiance, Threshold elevation angles, Seasonal affected disorder

ORAL PRESENTATION SESSION C

BREAKFAST TRYPTOPHAN CONTENT CORRELATES WITH MORNING-EVENINGNESS IN JAPANESE INFANTS

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Objectives: This study aims to clarify the relationship between breakfast contents, focused on tryptophan, and diurnal rhythm of Japanese infants.

Methods: The Japanese version of the M-E questionnaire originally constructed by Torsvall and Åkerstedt (1980) and another original questionnaire including questions on sleep habits (*e.g.*, bedtime, wake-up time, difficulty to fall asleep) and questions on breakfast contents: "Please mark all the foods taken by your child at breakfast from among eggs, meats [including ham and bacon], milk, vegetable, boiled-rice, bread, fruit juice, fermented soybeans, seaweed or laver, non-dried fish, dried-fish, coffee, tea, green tea, and soybean soup." Questionnaires were administered in June 2004 to 740 children aged 0 to 6 yrs attending one of nine municipal nursery schools in Kochi and their parents (638 mothers and 55 fathers, who answered the 2 questionnaires both on themselves and their children).

Results: Average and S.D. of M-E scores of infants and their parents was 20.03 ± 0.11 and 19.29 ± 0.14, respectively. The index of tryptophan taken in breakfast (Try-Index) was calculated from the tryptophan amount included in 100 g foods and standard amount per meal (Gomyo and Hasegawa, 1993). There was significant positive-correlation between M-E score and Try-Index (Pearson's test: $r = 0.180$; $p < 0.001$). The Try-Index of infants who had frequent difficulties of falling asleep or being awakened was significantly lower than those who did not have these difficulties (Kruskall-Wallis-test: fall asleep, $p = 0.027$; awakened, $p = 0.008$). The Try-Index of infants who had very short sleep-latency (< 5 min) was 440, which was significantly higher than that (302) of those who had a sleep–latency > 60 min (Kruskall-Wallis test: $\chi^2 = 14.719$, $df = 5$; $p = 0.011$).

Conclusions: Tryptophan ingested at breakfast is very important for children to retain a morning-type diurnal rhythm and high quality

sleep, presumably, through metabolism of tryptophan to serotonin during the daytime and melatonin at night.

Keywords Japanese infants, Breakfast contents, Tryptophan, M-E scores, Sleep quality

AGING EFFECTS ON NA^+-K^+, CA^{+2}, MG^{+2}-ATPASE ACTIVITIES IN ISOLATED SYNAPTOSOMES OF RAT BRAIN

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Objectives: Cerebral metabolism of glucose, one of the determinants of tissue ATP level, is crucial for CNS function. Recent evidence indicates that chronic hyperglycemia may inhibit synaptic Ca^{+2}-ATPase by glycation. The activity of P-type pumps, namely Na^+, K^+-ATPase, Ca^{+2}-ATPase, and Mg^{+2}-ATPase were examined in rat brain synaptosomes to determine both *in vivo* and *in vitro* if changes in the enzyme activity related to aging are associated potentially with alteration in glucose homeostasis.

Methods: Male Wistar rats kept under a12:12 L:D schedule with food and water *ad libitum* were sacrificed between 09:00 to 11:00 h by decapitation. Synaptosomes were isolated from brains of 5-d-, 3-m-, and 18-m-old-rats. The enzymatic activities of Na^+-K^+, Mg^{2+}, and Ca^{2+}-ATPases were expressed in μmol of Pi liberated from ATP by 1 mg of synaptosome protein during 1 h. Serum insulin by the RIA and glucose concentration by the glucose oxidase method was also measured. Student's *t*-test was used for statistical evaluation of mean values and differences were considered to be significant at a level of $p < 0.05$.

Results: The *in vivo* study demonstrated that 18 m-old rats are characterized by hyperglycemia, hyperinsulinemia, and elevated IRI/glucose ratio. These conditions had a different impact on the activities of ATPases tested *in vivo*: Na^+-K^+ ATPase activity was unchanged, activity of Ca^{2+}-ATPase decreased; whereas, that of Mg^{2+}-ATPase increased significantly. During the *in vitro* experiments, prior incubation of isolated synaptosomes with glucose in concentrations 4.5 to 6.5 mM (corresponding to normoglycemia *in vivo*), stimulated Ca^{2+}-ATPase activity; whereas, higher glucose concentrations (10.0 to 12.5 mM) significantly inhibited enzyme activity. In contrast, activity of Mg^{+2}-ATPase in synaptosomes from old rat brains was not inhibited *in vitro*, even at high glucose concentrations. This may explain the increased activity of this enzyme in old, hyperglycemic rats *in vivo* (average blood glucose concentration of 10.26 ± 0.30 mM).

Conclusions: It seems the changes in activity of different P-type pumps differ with aging, and that adaptation of specific ATPases to glucose homeostasis disturbances is not identical.

Keywords Aging, Hyperglycemia, ATPases, Brain, Synaptosomes

EFFECT OF GEOMAGNETIC ACTIVITY ON NOCTURNAL URINARY MELATONIN IN HEALTH AT 70°N?

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Objectives: Above the polar circle, the sun does not rise above the horizon for several weeks during winter. Factors other than light may affect variations in melatonin, including disturbances in geomagnetic field (GM), which are felt more strongly close to the poles than at lower latitudes. In saliva, we found earlier that if large enough, changes in GM decreased melatonin concentration. A possible influence of GM upon the urinary secretion of melatonin is examined herein.

Methods: Ten healthy males in Alta, Norway at latitude 70°N collected their nightly urine at different times of the year (55 samples total). Individual melatonin concentrations were determined and compared to the average local geomagnetic disturbance index (K) for the span matching the urine collection interval.

Results: A circannual component was not statistically significant by cosinor. A component with a period shorter than 1 yr was detected for both K (0.69 yr; 95% CI: 0.59–0.78 yr; ordering $p = 0.007$) and urinary melatonin (0.80 yr; 95% CI: 0.64–0.96 yr; $p = 0.023$, not corrected for multiple testing). At an intermediate period of 0.75 yr, the acrophases are more or less in antiphase between K and melatonin ($-226°$ *vs.* $-61°$; ref: 1 Jan 1999), as would be anticipated if a negative relation existed between melatonin and GM. For $K > 3$, the log-transformed melatonin values are negatively correlated with K ($r = -0.331$; $p = 0.032$).

Conclusions: When strong enough ($K > 3$), GM is associated with a decrease in urinary melatonin, in keeping with earlier results. Melatonin may thus be influenced by both non-photic and photic solar effects, notably near the poles.

Keywords Geomagnetic activity, Geomagnetic disturbance index, Melatonin, Circannual rhythm

DISTINCT RECEPTION OF MOONLIGHT AND OF VENUS RADIATION IN CONNECTION WITH 28 AND 280-DAY BIORHYTHMS

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Objectives: We explored cosmic radiations related to 28 (menstrual cycle) and 280 d (human gestation) biorhythms.

Methods: We analyzed the intensity, polarization, and duration of terrestrial moonlight. Our results are compared with normative catamenial data from the chronobiological literature. We also studied the characteristics of Venus radiation. In additional to the solar reflected radiation, Venus is the source of a distinguishing IR radiation, perceived on the surface of Earth. The calculated time-span of its defined reception is compared with the length of human gestation.

Results: We determined the reception duration of lunar light as the Moon's synodic period (29.5d) minus the interval of perturbed reception (1.5d = 95% of 29.5d) around the New Moon. 29.5d–1.5d = 28d is the average duration of the human menstrual cycle. Besides the 28d biorhythm (of L.H., erythrocytes, leucocytes, thrombocytes, *etc.*), a circabiseptan biorhythm is also known. The analogous trend of the time evolution of some women's biological parameters (prolactin hormone, fibrinolysis, cholesterol, etc) reported by different authors with the variation of degree of polarization of moonlight is of interest. Our calculated well-defined reception of Venus radiation equals $\frac{1}{2}$ the Venus synodic period (584d) minus the interval of perturbed reception (96% of 292d, explicitly 1d around the inferior and 11d around the superior conjunction) or 292d-12d = 280d, which is the average length of human gestation.

Conclusions: We hypothesize that the analyzed radiations may be implicated in the genesis of the corresponding biorhythms, according to the bioactive properties of the polarized light and of IR radiation.

Keywords Human menstrual cycle, Human gestation, Moonlight, Venus radiation, Polarization

SEASONAL MODIFICATION OF CIRCADIAN RHYTHM IN FREE-RANGING EUROPEAN MAMMALS

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Objectives: To determine how circadian rhythms are influenced by climatic and nutritional conditions in free-ranging mammals throughout the year and how they express stress or disease.

Methods: Activity and grazing behavior were recorded continuously by the storage telemetry system ETHOSYS® in Przewalski horse, wild sheep, red deer, and cattle living under natural conditions. Daily levels and day-night relations were developed from the activity and grazing plots throughout the year. From spectral analysis, Degrees of Functional Coupling (DFC) and harmonic parts were calculated as indicators of the internal harmonic coordination.

Results: All species were mainly light-active following a typical bigeminus pattern. Against climatic and nutritional variations over the year, the different species reacted with specific activity patterns. These seasonal variations were related to feeding strategy. Parasitic disease, transportation stress, cold, heat, and noise disturbed the typical daily rhythm, which resulted in lower harmonic parts and DFCs and/or changed daily levels and day-night relations.

Conclusions: Continuous records of activity and grazing can be used to identify normally species-specific environmental adaptations and detect stress and disease. Independent of different species-specific rhythmic patterns, DFCs and harmonic parts are approaches to quantitatively assess animal patterns.

Keywords Activity rhythm, Telemetry, Power spectrum, Stress, Disease, Free-ranging animals

50 Hz MAGNETIC FIELD EFFECT ON PERIPHERAL BLOOD

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Objectives: Mimicking common circumstances of modern life, the experiment evaluated environmental low-frequency electromagnetic field (MF) repercussions under modified nyctohaemeral cycles in rodents.

Methods: 8 groups each composed of 8 male adult Wistar rats were entrained to 12:12 h L:D cycles for 14 days. Then, for 14 weeks, except for the controls, different groups of animals were exposed to 7Gauss/50 Hz MFs, 8 h/day (07:00 to 15:00 h), under normal L:D cycles, constant darkness (D:D), or constant light (L:L). Two groups, entrained to L:D cycles, received a sulphonamidic immunomodulator (S8:62.5 mg/kg/day, at 10:00 h), alone and associated with MF, respectively. At the beginning and end of the experiment, blood smears were obtained. Leukocyte subsets were determined along with neutrophil (PMN) phagocytic capacity, and complement activity (UCH50). Statistical significance by Student (t)-test corresponded to $p < 0.005$. Concurrently, a histologic examination of brain, lymphatic organs, digestive tract, and testis was performed.

Results: PMN and lymphocytes decreased under L:L and D:D. MF associated to either L:L or D:D regimens enhanced PMN and lymphocyte drop. Under L:L + MF and D:D + MF, changes were significant ($p < 0.005$). S8 increased both PMNs and lymphocytes under L:D and L:D + MF, but to a lesser extent in irradiated animals. Eosinophils increased under L:L and D:D ($p < 0.005$). Under MF and illumination stress, eosinophilia was less important. Basophils increased under L:L and D:D, but only under D:D + MFs and L:L + MF in a statistically significant way. Monocytes also increased under L:L and D:D ($p > 0.005$). S8 diminished eosinophils, basophils and monocytes under both L:D and L:D + MF. UCH50 increased in all groups, reaching its highest value under L:L + MF ($p < 0.005$). PMN phagocytic capacity was augmented under L:D + S8, L:D + S8 + MF, D:D, and D:D + MF ($p < 0.005$). Under L:L and L:L + MF, weight gain was statistically significant. The histopathologic results paralleled hematologic data, expressing a statistically significant chronic inflammatory-like reaction. Occasionally, testis presented epithelial proliferative and hypersecretory areas.

Conclusions: Most evident changes occurred under L:L and L:L + MF conditions. MFs partially counteracted S8 activity. MFs and lighting (natural MF) stress exerted a cumulative effect, modulating electrochemical phenomena and activating the photo-neuro-immuno-endocrine axis.

Keywords Electromagnetic field, Immunity, Light-dark cycles, Blood cells

POSTER PRESENTATION SESSION D

THE PRECONDITIONING BY HYPOVENTILATION IS DEPENDENT ON THE TIME OF STUDY DURING THE 24H LD CYCLE IN WISTAR RATS

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Objectives: Hypoventilation, as one of ventilatory disorders, decreases the electrical stability of the heart similarly as ischemia. If preconditioning by short cycles of ischemia has a cardioprotective effect against harmful influences of a prolonged ischemic period, then preconditioning by hypoventilation should also have a similar effect. However, it is not known whether this effect is circadian rhythm-dependent. Thus the aim of study was to evaluate the changes of the electrical stability of the heart after preconditioning by hypoventilation as function of time with reference to the 24 h LD cycle.

Methods: The ventricular arrhythmia threshold (VAT) was measured in anesthetized female Wistar rats (ketamine/xylazine 100 mg/15 mg/kg, i.m., open chest experiments). The experiments were performed in two phases. In the first phase of experiments (light group), the animals were exposed to a 12:12 h LD cycle with the dark period from 18.00 to 06.00 h. In the second phase of experiments (dark group), the dark period was inverted, so it occurred from 06.00 to 18.00 h. The preconditioning (PC) was induced by one (1PC), two (2PC), or three (3PC) cycles of 5 min hypoventilation followed by 5 min reoxygenation. The parameters of hypoventilation- $V_T = 0.5$ mL/100 g, 20 breaths/min, and normal ventilation and reoxygenation -$V_T = 1$ mL/100 g, 40 breaths/min.

Results: In the light period (the first phase of experiments), 20 min hypoventilation significantly decreased the VAT only in the control and 1PC group ($p < 0.05$) *vs.* initial baseline values. The VAT was increased during hypoventilation ($p < 0.001$) and reoxygenation ($p < 0.001$) only in 3PC. In the dark period (the second phase of experiments), no statistically significant differences were found between initial baseline thresholds and VATs from hypoventilation and reoxygenation.

Conclusion: Cardioprotection against hypoventilation/reoxygenation induced decrease of VAT proved to be effective only after three cycles of preconditioning by hypoventilation. The rat myocardium appears to be less sensitive to systemic hypoxia induced by hypoventilation during the dark than light span (VEGA grant 1/0512/03).

Keywords Hypoventilation, Preconditioning, Wistar rat, Arrhythmias, Light-dark cycle difference in response to preconditioning

LIGHT-AT-NIGHT INCREASES GROWTH OF DMBA-INDUCED RAT MAMMARY ADENOCARCINOMAS

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Objectives: The goal of this work was to assess whether light contamination at night influences the rate of growth of DMBA-induced mammary adenocarcinomas as well as the role of melatonin in this process.

Methods: Female (55-day-old) SD rats received a single i.g. dose of DMBA. When mammary tumors (1 cm diameter) were palpated, the animals were exposed to one of the following patterns of lighting: **LD**, 12 h light (300 lux)/12 h darkness; **LL**, constant lighting (300 lux); **LD_{PLC}**, same as LD condition but applying a 30 min flash of light (300 lux) in the middle of darkness (PLC = punctual light contamination); **LD_{CLC}**, same as LD condition but maintaining a dim light (0.21 lux) during all the period of darkness (CLC = constant light contamination). The evolution of the tumors (size and number) plus survival of animals was recorded. Urine of a 24 h period was collected in two separate fractions (representing the light and dark spans, respectively) to assess sulphatoxymelatonin. At sacrifice, blood samples were collected for determination of serum estradiol.

Results: The lowest rate of tumor growth corresponded to rats under LD. Animals exposed to LL, LD_{PLC}, or LD_{CLC} pattern of lighting showed significantly higher rates of tumor growth than animals in LD. Since the 7th week of exposure to nocturnal light contamination, the average tumor size of rats in LD_{CLC} was significantly higher than in animals maintained under LD. The detection of differences of tumor size in relation to rats in LD takes 9 wks in the case of animals in LD_{PLC} and 11 weeks in the case of those under constant light (LL). Animals placed under LD_{CLC} had the lowest survival probability. There were no significant differences in terms of life span among controls (LD) and rats exposed to LD_{PLC} or LD_{CLC}. As expected, animals under a LD cycle show a clear day/night rhythm of sulphatoxymelatonin excretion; this diurnal rhythm disappears in animals exposed to light during the dark span (LL, LD_{PLC}, or LD_{CCL}). Animals exposed to dim light contamination at night (LD_{CCL}) showed serum concentrations of estradiol significantly higher than rats kept under LD, LL, or LD_{PLC}.

Conclusions: Light contamination at night increases the growth of chemically induced mammary adenocarcinomas. The constant contamination of darkness with dim light results in faster tumor growth and higher mortality than the other patterns of light contamination. The circadian and endocrine disruption induced by light contamination, with low nocturnal melatonin secretion and high serum concentration of estradiol, could explain the tumor growth. The answer to why the effects of nocturnal dim light contamination on carcinogenesis seem to be stronger than exposure to LL or LD_{PLC} requires future study.

Keywords Mammary cancer, Light-dark cycle, Light contamination of dark span, Sulfatoxymelatonin, Estradiol, Cancer animal model

Work supported by the Spanish MCYT (BFI2003-06305) and Institute of Health "Carlos III" (PI042603).

REACTION OF RATS CERVICAL LYMPH NODES AFTER PINEALECTOMY

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Objectives: The pineal gland acts as a relay where anabolic activity is replaced by catabolic activity and to adapt a biological rhythm according to the requirement of the organism. It is established that pineal extract stimulates lymphopoiesis in lymph nodes, and the focus of this study was the exploration of lymph nodes reaction four weeks and two months after pinealectomy using stereological methods.

Methods: 30 rats of both sexes was sham-pinealectomized (chirurgic treatment without pineal extirpation and 30 other animals were pinealectomized (with pineal extirpation). The animals were kept under the same ecologic condition and water given *ad libitum*. Animals of the each group were sacrificed the same day between 10:00–14:00 h to equalize the effect of hormonal rhythms. The paraffin sections were stained with PAS-alcian blue, using HE and Bielschowsky methods. For stereological analysis, the Weibls multipurpose test system (M-42) was applied. The volume density (Vv) of nodal structure and numerical density (Nv) of lymphocytes and relative cellular distribution was evaluated.

Results: At 4 wks after treatment, cortical tissue of lymph nodes was reduced after ablation of pineal gland, particulary the paracortical regions. A decreased number of lymphocytes was observed in the cortex and medullary cords only in female rats. Stereological analysis confirmed that increased volume density of medulla is result of medullar sinus dilatation. Under regressive changes of cortical structures can be considered the involuntary processes in germinal centers and reduction of younger form of lymphocytes. Macrophages were activated and increased PAS-positive inclusions were observed at the cortico-medullary junction and in medullary cords. Reticular stroma were intensively impregnated, with fibrills more coarsed and fragmented. Two months after pinealectomy, the reactive changes in lymph nodes were in a contrary direction compared with those seen at 4 wks. Cortical volume density was increased and macrophage stimulation was prominently evident only in pinealectomized female rats. Mastocytes were more numerous in both sexes of the

pinealectomized animals at 4 wks but at 2 months involuntary changes was observed.

Conclusions: It is evident that two phases of lymph node reaction after pinealectomy exist. In the first an involuntary one, and in the second a reactive stability one. It is also to be striking that sex differences exist in lymph nodes reaction after pinealectomy.

Keywords Lymph nodes, Pinealectomy stereology, Rats

MEASUREMENT OF CIRCADIAN EFFECTIVE RADIATION OF NATURAL AND ARTIFICIAL SOURCES

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Objectives: Reliable measurements of circadian effective irradiance and radiant exposure (dose) for daily application.

Methods: Two principle methods are applicable: 1) The spectral irradiance $E(\lambda)$ of an exposed area is measured by using a spectral radiometer. By calculation, the spectral irradiance $E(\lambda)$ is weighted with the action spectrum $s_{circ}(\lambda)$ of melatonin suppression, and consequently one gets, by integration, the circadian effective irradiance:

$$E_{circ} = \int E(\lambda)S_{circ}(\lambda)d\lambda$$

This is an accurate method for measuring circadian effective irradiances. However: the spectral radiometer equipped with a double mono-chromator, being necessary for this kind of measurement, is expensive and not designed for easy transportation. 2) Integral measurement by using a receiver, whose spectral sensitivity $s_{rec}(\lambda)$ is well adjusted to the action spectrum $s_{circ}(\lambda)$. This method allows one to measure the circadian effective irradiance E_{circ} directly. The poster shows technical details of the instrument which is inexpensive, light, and easy to handle. The measured E_{circ} values are in line with the results of method 1 in a range of $\pm$ 15%, being sufficient for practical purposes.

Results: Method 1 is useful for scientific application and calibration, while method 2 is useful for daily application, *e.g.*, for studies in medicine and industrial medicine.

Conclusions: The new instrument following method 2 is useful, for instance, for measuring threshold values for circadian effective irradiance

and radiant exposure (dose), and for correlation studies between circadian effective radiant exposure and the daily behavior of groups of people, or monitoring the circadian effective exposure of people at workplaces.

Keywords Circadian effective radiation measurement, Action spectrum, Melatonin suppression, Industrial medicine

MELATONIN (M) PROFILES IN PATIENTS WITH BRAIN TUMORS-PRELIMINARY STUDY

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Objectives: M secretion is one of the best rhythm markers for the circadian clock. To function as a circadian pacemaker, the suprachiasmatic nucleus (SCN) must receive photic information via the retino-hypothalamic tract (RHT). Surgical resection of the RHT, leaving the optic nerve intact, results in free-running of rhythms. Abnormal M circadian rhythms have been reported in pts with pituitary tumors of different origin. The aim of this study is to check the M profile in pts suffering from brain tumors localized on the neuronal pathways between the retina–hypothalamus and pineal gland.

Methods: 4 pts with brain tumors underwent examination. All pt medications were discontinued 2 days before hospital admission. 3 had pituitary gland tumor and were awaiting surgical operation (Operatio transnososeptosphenoidalis, Excochleatio et aspiratio tumoris). One who had a brain tumor (medulloblastoma) was cured by craniotomy. In each case, clinimetric examination, MRI of brain, and laboratory tests were performed. Conventional measurement of heart rate, blood pressure, and body temperature was conducted before blood sampling. The level of blood M was measured at 22:00, 02:00, 06:00, and 10:00 h. Pts were kept in a dark isolated room and in horizontal position for a 12 h period. The Melatonin IBL ELISA (Hamburg) method was used to determine the serum blood M concentration. The minimum detectable concentration of M was 3 pg/mL. The intra-assay precision varied from 3.5to 10.5%. The inter-assay precision varied from 9 to 19%. The acrophase (peak time), DMLO (dim light M onset), and morning slope in M were studied.

Results: In the case of a pt (47-yr-old woman) suffering from tumor of the anterior pituitary secreting ACTH, a significant "flattening" of the M profile with concomitant circadian phase advance was observed. A 64-yr-old woman with pituitary tumor secreting prolactin and exerting pressure at the optic chiasm exhibited an abnormal, almost linear melatonin profile. Surprisingly, a pituitary tumor secreting TSH found in 49-yr-old patient did not affect the M profile in spite of its rather large size. On the other hand, medulloblastoma in a 23-yr-old woman produced circadian phase advance and decreased amplitude of the M secretion profile.

Conclusions: Our results indicate that the relationship among localization, tumor size, and abnormalities in M profiles is rather complicated and may be affected by the kind of hormone secreted by the pituitary tumor. These preliminary results require further complementary studies with increased number of pts.

Keywords Melatonin, Brain tumors, Circadian rhythms, Hunan beings

POST-EMBRYONIC CHANGES IN THE DIURNAL RHYTHM OF CHICKEN PINEAL AA-NAT ACTIVITY

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Objectives: The aim of this study was to examine in chickens the post-embryonic changes in the circadian rhythm of the activity of pineal aryl-alkylamine N-acetyl-transferase (AA-NAT), a key enzyme in the melatonin biosynthetic pathway.

Methods: Experiments were performed in the middle of winter on male Hi-Line chickens obtained from a local commercial hatchery. On the day of hatch, chickens were transported to the Faculty animal facilities and reared under controlled light (L:D = 12:12 h) and temperature conditions (32 ± 2°C during the first week and 24 ± 2°C thereafter), with free access to standard food and water. On days 2, 9, and 16 after hatch, chickens were killed at 2 h before and every 1 to 2 h during darkness. Pineal glands were isolated under dim red light, immediately frozen in liquid nitrogen, and stored at −85°C prior to analysis of AA-NAT activity, assessed by the liquid biphasic diffusion method.

Results: In all chickens examined, regardless of age, NAT activity increased in the dark phase. It was lowest in youngest birds and increased

with age, reaching a peak in the middle of darkness in the oldest birds examined. This result is similar to that observed by us previously in 3-wk-old male chickens, kept in winter in the same laboratory conditions.

Conclusions: Pineal AA-NAT activity in chickens exhibits a circadian rhythm changing during post-embryonic development. It responds to the environmental lighting conditions already in the first day after hatch, but its seasonal feature develops few days later.

Keywords AA-NAT, Pineal, Melatonin, Diurnal rhythm, Chickens

Supported by State Committee for Scientific Research grant 2 PO4C 080 27

THE ORAL GLUCOSE TOLERANCE RHYTHM OF YOUNG HEALTHY SUBJECTS IS CHRONOTYPE-DEPENDENT

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Introduction and Objectives: Previous studies have shown that oral glucose can be absorbed and metabolized with different effectiveness depending on the time of the day at which it is ingested. However, despite the obvious interest of this observation for carbohydrate metabolism in people with a nocturnal lifestyle, it is not known how the morning-evening chronotype may affect this rhythm. The aim of this study is to determine whether time-of-day differences in the oral glucose tolerance test (OGTT) are related to human chronotypes in young healthy subjects.

Methods: OGTTs were given to 33 non-obese, young (19 to 20 yrs) subjects, 15 men and 18 women. They received two oral administrations of glucose (50 mg in 200 mL tap water) at 09:00 and 20:00 h, on different days. Each test was preceded by an 11 to 12 h fast. Blood samples were taken at 30 min intervals for the following 2 h. The Horne and Östberg Morningness-Eveningness Questionnaire was used to asses the morningness/eveningness chronotype of each subject. The score obtained in the test was correlated with Tmax, Cmax, and area under the OGTT curve (AUC).

Results: Our results indicate that glucose tolerance (expressed as AUC) is significantly reduced in the evening compared to the morning test ($p < 0.00003$). For the morning OGTT, glucose tolerance increased along with the morningness score ($p < 0.05$). Conversely, in the case of the evening OGTT, although not significantly, glucose tolerance tended to increase as the eveningness score did. No relationship was observed between test score and Tmax or Cmax.

Conclusions: The present research reveals that glucose tolerance is affected by the person's chronotype. Morning types tend to show good tolerance in the morning and impaired tolerance in the evening, whereas this trend is reduced or entirely absent in the evening type.

Keywords Glucose overload, Glucose tolerance, Circadian rhythms, Humans, Chronotype, Morningness-eveningness

PLASMA MELATONIN AND DHEA IN ADULT AND AGED OCTODON DEGUS

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Introduction and Objectives: Since their secretion is known to decline with age, plasma melatonin and DHEA levels have been proposed as endocrinological biomarkers of aging in mammals. In addition, a reduction of the nocturnal to diurnal melatonin secretion ratio appears to be an aging feature in most species. *Octodon degus* is a long-lived hystricomorph rodent from central Chile that can suffer from spontaneous degenerative diseases and, unlike the rat, is a diurnal animal. Despite its interest as an animal model for chronobiological, chronopharmacological, and aging studies, no information is available to date regarding its plasma melatonin and DHEA levels. Thus, the aim of this work is to evaluate mid-light (ML) and mid-dark (MD) plasma melatonin and DHEA levels in mature *vs.* old degus.

Methods: *Octodon degus* of both sexes were divided according to their age in "adults" ($n = 11$, 32 ± 3 months old) and "aged" ($n = 7$, 57 ± 1 months old). They were kept individually housed in isolated chambers under 12:12 LD (lights on at 08:00 h) and constant temperature (22 to 24°C). Blood samples were obtained by jugular venipuncture at MD and ML. Samples were taken at the beginning of the study and after 5 months. Plasma melatonin and DHEA levels were analyzed using a commercial RIA kit (IBL Hamburgo®, Hamburg, German) and ELISA (IBL Hamburg®), respectively.

Results: No significant plasma melatonin or DHEA differences were detected between sexes. However, the MD-to-ML relative amplitude and mean melatonin values were reduced in aged animals, due both to a significant nocturnal melatonin concentration reduction and a slight diurnal secretion increase. As regards DHEA levels, no reduction in

MD-to-ML amplitude or mean values could be observed with aging in this species.

Conclusions: Our preliminary results confirm, for the first time, an age-related decline of melatonin levels in *Octodon degus*, as has been described in nocturnal rodents and in human beings. This decline is mainly due to impaired nocturnal melatonin secretion. In humans, DHEA levels gradually decrease with age but, surprisingly, no age-related DHEA changes were observed in the degus. Further studies including young animals are needed to confirm this latter result.

Keywords *Octodon degus*, Melatonin, Aging, DHEA, Circadian rhythms

This work was founded by a grant from The Seneca Foundation (PB/43/FS/02)

TRANSCRANIAL MAGNETIC STIMULATION AT pT AND SYNCHRONIZATION OF THE ALPHA RHYTHM

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Objectives: To obtain an electroencephalographic response and to evaluate qualitatively the re-distribution of cerebral rhythms in healthy subjects (pattern for normality) by employing physiological transcraneal magnetic stimulation (TMS) at **10 pT** and **8 Hz.** The patterns are to be compared in further research, with applications carried out in chronomedicine.

Methods: We have employed digital EEG, mod.SAM 32 FOFC 1, with the latest version System PLUS Software by Micromed, and spectral analysis by Fast Fourier Transformed, which incorporates frequency maps. We have also used a prototype TMS stimulator, mod. MG28TR10, which incorporates a timer to control, in the EEG registry, the stimulation periods and pauses (1.5 min followed by a 1 min pause). The sample was made up of 16 volunteer healthy subjects, (8 male and 8 female) properly and ethically informed about the experiment. The records were registered inside a Faraday chamber under controlled electromagnetic time-space conditions with the subjects comfortably seated with eyes open and without outside stimulation. A basal EEG

was first carried out for 5 min followed by a 10 min long sequence stimulation.

Results: A global increase in the Alpha and Beta rhythms and decrease in Delta and Theta rhythms were observed. Alpha and Delta registered the highest increase and decrease, respectively. The most relevant increase of the **alpha rhythm** during stimulation was registered by the frontal electrodes **Fp1, Fp2, F3**, and **F4**, as well as by the central **Cz**. This leads us to believe that under stimulation there is a displacement of the physiological alpha rhythm towards the anterior brain areas. (Figures 1 and 2).

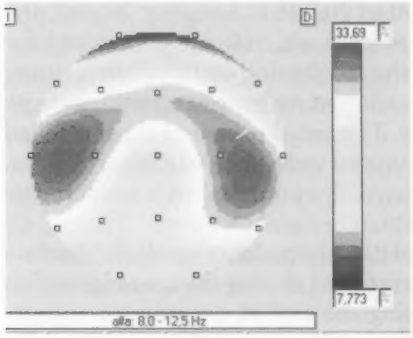

FIGURE 1 Basal state of alpha rhythm.

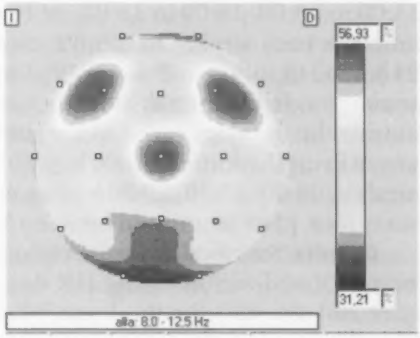

FIGURE 2 Redistribution of alpha rhythm under TMS.

Conclusions: By means of the TMS at 10 pT and 8 Hz, we obtained a mimetic global neuronal bioelectrical response. We also found a displacement of the alpha rhythm towards the frontal and central areas of the brain.

Keywords Magnetic Stimulation, EEG, Brain rhythms

We thank Siemens, S.A. for its collaboration and sponsorship.

ENTRAINMENT OF THE HEART RATE (HR) CIRCADIAN RHYTHM INDUCED BY PHYSICAL EXERCISE

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Objectives: Knowledge of the time course of variables tied to physiological modifications induced by physical exercise is important in sport; indeed, the training could represent an important entraining factor that influences the circadian system and consequently performances. Of particular interest is the evaluation of the circadian rhythm of HR in power and endurance disciplines, for the effect that physical exercise can have on the modulation of the temporal HR trend. The purpose of this study was to investigate how training, done at different circadian times, can modify the synchronization of the HR circadian rhythm.

Methods: The study was done on 20 male endurance athletes that adhered to 5 weekly 2 h daily training sessions for an overall period of 4 wks. They followed a different weekly training timetable (09:00 to 11:00, 11:00 to 13:00, 16:00 to 18:00, or 18:00 to 20:00 h), changing, in turn, the time slot each week. On the 5th day of each wk, HR was monitored for 24 h by a HR monitor (Polar S810). On the monitoring day the daily training session was held as usual. Subjects were allowed no further sporting activity outside the training session of the study. The subjects maintain a controlled way of living, bedtime at 23:00 h $\pm$ 30 min and wake up at 07:00 h $\pm$ 30 min, meals at 08:00, 13:30, 20:30 h; they were daily controlled by a trainer. The study took place in autumn between September and October.

Results: Statistical analyses employed the single cosinor method. The data were analyzed excluding the HR data recorded during the training session span and the data for the hour following the end of the training session. The HR circadian rhythm was statistically significant ($p < 05$) in all the training session times. The mean cosinor analysis of the data collected during the 4 training wks demonstrated a statistically significant difference in acrophase (peak time of circadian HR rhythm). The acrophase was progressively postponed during the afternoon: the HR acrophase for training done between 18:00 and 20:00 h was delayed approximately 3 h compared to that when training was done between 09:00 and 11:00 h.

Conclusions: The statistically significant difference between the circadian acrophases of the 24 h HR patterns of the 4 training session wks suggest that the entrainment of the HR circadian rhythm can be achieved by physical exercise.

Keywords Entrainment, Heart rate, Physical exercise, Circadian rhythm

DEVELOPMENT OF BIOCHIPS FOR CHRONOBIOLOGIC INVESTIGATIONS

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Objectives: Different tumor markers are valuable in the diagnostics and monitoring of cancer pts. Tumor marker combination assay is more sensitive and specific than the estimation of one marker. The chronobiologic approach to tumor marker study has much potential for yielding information about tumor development, but investigations of several markers around-the-clock or month are too costly to conduct. Application of a new microchip technique makes it possible to test samples using many tumor markers. The goal of the study was to develop an on-chip immunoassay for a series of the wide-spread tumor markers, including carcinoembryonic antigen (CEA, marker of colon cancer); prostate specific antigen (PSA, the marker of prostate cancer); α-fetoprotein (AFP, marker of liver cancer); and carbohydrate antigens CA-125, CA-15-3, and CA 19-9 (markers of ovarian, breast, and pancreatic cancer).

Materials and Methods: Gel-based microchips with immobilized monoclonal antibodies against tumor antigens were manufactured. Antigen-capture two-site ("sandwich") assay was used for the analysis. Microchips were treated with solutions of antigen or blood sera and reaction was detected by fluorescently labeled second antibodies; fluorescence signals were recorded by fluorescent microscope. Fluorescence intensities were plotted versus tumor marker concentrations to measure antigens in blood serum samples.

Results: It was demonstrated that the possibility exists for determination of small concentrations of tumor markers by fluorescent immunoassay by the chip conditions. The sensitivity of the PSA (both total and free) assay was 0.1 ng/mL, CEA–1 ng/mL, and AFP–5 IU/mL. The correlation coefficient between the data for sera PSA content of prostate cancer pts obtained by biochip analysis and by PSA/Total EIA II Cobas Core kit (Switzerland) was 0.99 ($p < 0.01$).

Conclusions: The application of gel-based biochips for evaluation of different tumor markers opens the possibility for the analysis of a variety of samples of pt sera using many tumor markers. The method allows one to miniaturize the analysis and to decrease significantly the amount of analyzed sample. This extends considerably the capabilities of chronobiological investigations.

Keywords Protein biochip, cancer diagnostics, tumor markers

INFLUENCE OF PHOTOPERIOD AND MELATONIN ON EXPERIMENTAL PERITONITIS IN SIBERIAN HAMSTERS

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Objectives: This study examined in the seasonal breeder, Siberian hamster (*Phodopus sungorus*), the influence of photoperiod (long day *vs.* short day) on the development of peritoneal inflammation and activity *in vitro* of selected immune cells in presence or absence of melatonin.

Methods: Adult male hamsters were housed under either 16L:8D (long day, LD) or 8L:16D (short day, SD) photoperiods for 6 to 8 weeks in constant temperature (22°C) with free access to standard food and water. Experimental peritonitis was evoked by a single injection of zymosan (2 to 5 mg/kg body weight). Animals were killed at specific time points after the injection and peritoneal leukocytes (PTL), testicular macrophages (TM), and splenocytes were isolated for *in vitro* assays, in which the effect of melatonin added in a wide range of concentrations was examined. PTL and TM activity was assessed with the use of fluorescent dye CM-H_2DCFDA. Splenocyte proliferation was measured by ^{3}H-thymidyne incorporation assay.

Results: In zymosan-injected hamsters, the activity of PTLs was increased compared to intact animals, and this effect was much more pronounced in LD. Melatonin added to cell suspensions significantly inhibited PTL activity isolated form animals housed under both photoperiods. Zymosan-induced peritonitis had no effect on TM activity; however, it inhibited splenocyte proliferation.

Conclusions: Experimental peritonitis in SD hamsters is less evident than in LD animals. Also, splenocyte proliferation in SD is lower than in LD. These effects may result form the immunomodulatory properties of melatonin.

Keywords Hamster, Peritonitis, Photoperiod, Melatonin

Supported by State Committee for Scientific Research grant 2 PO4C 090 27

CIRCADIAN RHYTHM IN BLOOD PRESSURE (BP) AND HEART RATE (HR) OF HEALTHY INDIANS

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Objective: We examine the circadian rhythm of BP and HR of healthy Indian subjects.

Methods: Fifty apparently healthy subjects aged 18 to 25 yrs (31males and 19 females) were studied. Ambulatory BP was monitored (ABPM) for 24 h by A&D TM-2430 (A&D Co., Japan). The device was programmed to take measurements every 15 min from 07:00 to 22:00 h and 30 min from 22:00 to 07:00 h. Ambulatory BP measurement of each subject was done on work-days and within a time-span of 3 months. Data were transferred to a computer and the averages of the systolic BP (SBP), diastolic BP (DBP), and HR were calculated for the daytime, nighttime and 24 h periods.

Results: Data were analyzed by the Halberg Chronobiology Centre (University of Minnesota, MN, USA) by rhythmometric (Cosnior) analysis. Conventional data analysis was done by he authors and identified the following reference (mean and SD) values: 24 h SBP = 117 ± 8 mm Hg; 24 h DBP = 73 ± 6 mm Hg; and 24 h HR = 85 ± 8 beats/min. Similarly, daytime values were 119 ± 10 mm Hg for SBP; 74 ± 6 mm Hg for DBP; and 88 ± 8 beats/min for HR. Nighttime values were 102 ± 10 mm Hg for SBP; 61 ± 7 for DBP; and 68 ± 7 beats/min for HR. Daytime values were found to be significantly higher (p value < 0.001) by 'paired t test'. Percent dip in SBP was 14.3 ± 2.1%; 17.5 ± 2.3% in DBP; and 22.72 ± 5.8% in HR.

Conclusions: Although the accurate conclusion can only be drawn on availability of rhythmometric analysis, the results of this study can serve as a preliminary reference standard for the time-specified values of BP and HR in healthy Indian subjects.

Keywords Ambulatory BP measurement, Indians, Circadian rhythm, Blood pressure, Heart rate

CIRCADIAN VARIATION OF BLOOD PRESSURE (BP) IN DIABETIC PATIENTS (PTS) WITH AUTONOMIC NEUROPATHY

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Objectives: Diabetes is a chronic debilitating disease that poses a high risk to pts of developing cardiovascular morbidity. This study elucidates the circadian BP pattern of Indian diabetics.

Methods: The study was conducted on 30 normotensive diabetic pts and 20 age and sex matched non-diabetic controls. Pts were selected from the Outpatient Department of Gandhi Memorial and Associated Hospital, Lucknow. 24 h ambulatory BP measurement (AMBP) and heart rate (HR) were conducted by a noninvasive ambulatory monitor (A&D TM-2430; A&D Co., Japan); variations in R-R interval and postural changes in BP were also recorded. The indices were assessed for correlation with various signs of autonomic neuropathy, including coefficient of variation of R-R interval and orthostatic hypotension.

Results: Difference in the average BP during daytime and average BP during nighttime was reduced in diabetic pts; this difference was statistically significant ($p < 0.05$) between control and study pts. The normal dipping pattern in the BP seen in control subjects was significantly reduced in diabetic pts with autonomic neuropathy.

Conclusion: 24 h ABPM can detect variation in the circadian BP pattern and is useful in evaluating diabetic autonomic neuropathy.

Keywords AMBP, Circadian rhythm, Blood pressure, Diabetes, Autonomic neuropathy

RHYTHM OF MELATONIN AND INTERLEUKIN-1B IN FORMULA-FED INFANTS

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Objectives: Melatonin is known to exert significant influence on different cytokines and on the sleep/wake rhythm in the newborn. The objective of the present study was to determine the rhythms of melatonin and interleukin 1-B in infants fed formula milk.

Methods: The study was done on 10 human infants of 8 weeks of age ($n = 10$) being fed formula milk, the ingredients per 100 g being: 12 g protein, 58 g carbohydrates, 26 g lipids, 2 g minerals, 32 mg taurine, and

17 mg L-carnitine (Ordesa S.L., Spain). Feeding was timed at intervals of 4 h. Urine was collected from nappies over a 24 h period for determination of 6-sulfatoxymelatonin and interleukin-1B assayed by ELISA and ELISA commercial kits (IBL and Bhulman, respectively). A non-parametric Kruskal-Wallis ($p < 0.05$) statistical test was performed to assess differences in mean values (SSPS.11.5 software package).

Results: 6-sulfatoxymelatonin peak during the light (06:00 to 18:00 h) period; levels were lowest during the dark (18:00 to 06:00 h) period, indicative of a circadian rhythm of melatonin. The difference in the mean level of interleukin-1B during the light (38.04 $\pm$ 2.82 pg/mL) and dark (27.85 $\pm$ 2.5 pg/mL) period were not statistically significant.

Conclusions: Formula-fed infants exhibited a circadian rhythm of melatonin. This is an important sign of the regulation of the sleep/wake rhythm. Interleukin-1B levels were similar throughout the 24 h. Formula milk did not alter the development of the melatonin circadian rhythm in our sample of newborns.

Keywords 6-sulfatoxymelatonin, Interleukin-1B, Infants, Formula milk

COMPARATIVE STUDY OF THE SLEEP/WAKE RHYTHM IN BREAST-FED AND FORMULA-FED INFANTS

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Objectives: It is known that breast milk is the best for the development of infants throughout the lactation period. Natural milk is necessary for the immune system and other biological mechanisms. However, is not known if breast milk affects the sleep/wake rhythm.

Methods: The study involved 10 breast-fed only and 10 milk formula-fed only infants. The formula's ingredients per 100 g were: 12 g protein, 58 g carbohydrates, 26 g lipids, 2 g minerals, 32 mg taurine, and 17 mg L-carnitine (Ordesa S.L., Spain). Feeding was at intervals of 4 h in both groups of infants. Data from an actiwatch attached around the leg was analyzed with the *Sleep Analysis* software package (Cambridge Neurotechnology) for the sleep parameters of: Time in Bed, Assumed Sleep, Real Sleep, Efficiency of Sleep, and Latency of Sleep. A non-parametric Kruskal-Wallis ($p < 0.05$) statistical test was performed to assess the significance of mean differences (*SSPS.11.5* software package).

Results: Breast-fed infants showed significantly ($p < 0.05$) greater Assumed Sleep, Real Sleep, and Efficiency of Sleep than formula-fed infants.

Conclusions: The results indicate that breast milk is advantageous during lactation for the establishment of the sleep/wake circadian rhythm.

Keywords Sleep, Infants, Formula, Breast milk, Sleep-wake circadian rhythm

EFFECT OF MORNING VERSUS EVENING TRANDOLAPRIL ADMINISTRATION IN HYPERTENSIVE PATIENTS: A PRELIMINARY STUDY

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Objective: Time of drug administration is one of the factors influencing drug efficacy. This study explored the administration-time-dependent antihypertensive efficacy of trandolapril, an angiotensin converting enzyme inhibitor, by ambulatory blood pressure (BP) monitoring (ABPM).

Methods This was a single-blind, crossover trial of 11 previously untreated grade I or II hypertensive patients (mean $\pm$ DS: age 51.4 $\pm$ 2.6 yrs; 3 men and 8 women) treated for 3 months with a single daily dose of trandolapril (2 mg/day), either in the morning or evening. Each pt was monitored 3 times (baseline, after 12 wks of morning treatment, and after 12 wks of evening treatment) by 24 h ABPM (Spacelabs 90207) at 20 min intervals from 06:00 to 22:00 h and at 30 min intervals during rest of the day. No wash-out period was applied between the morning and evening administrations. All pts were normal dippers. Data were analyzed by ABPM-FIT and CV-SORT, and group comparisons were done by paired Student's *t*- test.

Results: There were no statistically significant differences between morning and evening trandolapril administrations in terms of the mean 24 h, mean daytime, or mean nighttime BP values. Mean 24 h systolic and diastolic BP (SBP/DBP) for baseline, morning, and evening administrations were: 144/92, 136/86, and 138/88 mm Hg, respectively. Heart rate (HR) was significantly reduced by morning, but not evening administration. Morning administration resulted in significant reduction in mean daytime DBP (96.3 to 89.7 mm Hg; $p = 0.02$), and evening administration resulted in significant reduction in mean nighttime DBP (85.8 to 78.8 mm Hg; $p = 0.01$). Means of 24 h, daytime, and nighttime rate-pressure

product (RPP: HR × SBP) were significantly reduced by morning trandolapril treatment; whereas, evening trandolapril treatment only reduced the nighttime values.

Conclusions: Trandolapril administered either in the morning or evening markedly reduces SBP and DBP. Morning administration seems preferable because it reduces DBP and RPP more effectively than evening administration.

Keywords Trandolapril, ABPM, Chronotherapy of hypertension, Chronopharmacology

EFFECTS OF EXOGENOUS MELATONIN ON THE CIRCADIAN SYSTEM OF AGED *OCTODON DEGUS*

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Objectives: Circadian rhythm amplitude reduction and phase instability are very common features of aged circadian systems. In most species, aging is associated with a decline in nocturnal plasma melatonin level; consequently, exogenous melatonin administration has been proposed to reverse some age-related processes. This research explored whether exogenous melatonin improves body temperature and wheel running activity circadian rhythms in a diurnal rodent, *Octodon degus*.

Methods: *Octodon degus* ($n = 18$, 32 to 60 months of age) kept under a 12:12 LD routine were subjected to 4 experimental phases. Following a 14-day baseline period, during which animals were allowed to eat and drink *ad libitum* (control period), their access to water was restricted to 2 h before darkness for 15 days (VEH), after which they were administered melatonin (MEL, 500 μg/kg body weight) dissolved in their drinking water, also under restricted access, for 15 additional days. Finally, animals were allowed to drink *ad libitum* again for another 14 days. Body temperature (BT) was recorded every 60 min by a surgically implanted intraperitoneal data logger (ThermoChron®, Dataloggers iButton, IDC S.A., Spain). Wheel running activity (WR) was recorded every 10 min.

Results: Water restriction (VEH) induced an amplitude and phase stability increase of the WR and BT circadian rhythms without affecting the phase angle of entrainment. Exogenous MEL caused an additional circadian amplitude increase, due to a pronounced BT drop; however, no changes in phase stability or angle of entrainment were detected compared to VEH. Surprisingly, these effects persisted for at least 2 wks after the cessation of treatment. Additionally, MEL administration, but not VEH,

suppressed the periodic WR increase associated with the female degu estrous, which reappeared once again after MEL treatment was removed.

Conclusions: Administration of exogenous melatonin dissolved in drinking water is able to reverse some aging-related circadian rhythm impairments, and these effects persist for several days after the termination of melatonin treatment. Moreover, melatonin seems to be an antigonadal hormone in female degu.

Keywords *Octodon degus*, Circadian rhythm, Body temperature, Estrus, Wheel running

This work was founded by a grant from the Seneca Foundation (PB/43/FS/02)

GSK3β AND THE MOLECULAR CLOCK AS NOVEL TARGETS FOR LITHIUM SALTS AND CLINICAL CHRONOTHERAPEUTICS

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Objectives: The molecular mechanisms driving the biological clock in the suprachiasmatic nucleus of the hypothalamus may play a role in mood disorders. A single nucleotide polymorphism (SNP)(− 50 T/C) falling into the effective promoter region (nt −171 to +29) of the gene coding for glycogen synthase kinase 3-β (GSK3-β) is known to regulate circadian rhythms in Drosophila. GSK3-β codes for an enzyme which is a target for the action of lithium, the inhibition of which causes antidepressant-like behaviors in a preclinical model. This study addresses the effect of this polymorphism on the age at onset of bipolar disorder type I, acute response to total sleep deprivation, and the therapeutic response to lithium salts.

Methods: A homogeneous sample of 333 Italian bipolar disordered pts was genotyped to analyze age at onset. We also studied the effect of this polymorphism on the acute response to total sleep deprivation of 60 depressed bipolar type I inpts. The effect of this polymorphism on the therapeutic response to lithium salts was assessed in 88 bipolar type I pts. Data about recurrence rate of mood episodes were collected for at least 2 yrs before and 2 yrs after lithium treatment.

Results: Age at onset was retrospectively ascertained with best estimation procedures. Homozygotes for the wild variant (T/T) showed an earlier age at onset than carriers of the mutant allele (respectively: T/T $n = 158$, mean $\pm$ SD age at onset 29.93 ± 8.61; T/C $n = 140$,

33.59 ± 11.77; and C/C $n = 35$, 35.43 ± 11.08), with the difference being highly significant ($F = 6.78$, df. 2,330; $p = 0.0013$). Homozygotes for the mutant allele of GSK3-β promoter (50 T/C) SNP showed a later onset of bipolar illness and better acute effects of total sleep deprivation treatment on perceived mood (as rated by visual analogue scales). Overall, these observations suggest a protective role for this genotype in respect to bipolar illness. Results showed also that homozygotes for the wild variant did not change their recurrence index after at least 2 yrs of lithium prophylaxis, while carriers of the mutant allele improved. These findings support the hypothesis that GSK is a target for the therapeutic action of lithium.

Conclusions: Results warrant interest for the variants of genes pertaining to the molecular clock as possible endophenotypes of bipolar disorder and for GSK3-β as a target of a new class of antidepressant drugs

Keywords GSK3-β, Sleep deprivation, Lithium, Bipolar disorder, Genotyping, Suprachiasmatic nucleus, total sleep deprivation treatment

CHANGES IN RIGHT DLPFC (BA9) NEURAL RESPONSES PARALLEL RESPONSE TO SLEEP DEPRIVATION IN BIPOLAR DEPRESSION

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Objectives: Chronic incapacity to suppress negative emotion may be a key factor in the genesis of depression. Studies of the neural correlates of transient normal sadness and healthy voluntary suppression of negative affect revealed a role for the right dorsolateral prefrontal cortex (DLPFC, BA 9). Total sleep deprivation (TSD) causes rapid antidepressant effects in pts affected by a major depressive episode, in the absence of confounding factors due to pharmacotherapy. This study explored the hypothesis that the antidepressant response to TSD is paralleled by changes in neural response of right BA9 to emotionally tuned stimuli.

Method: Gradient echo echo-planar imaging at 3.0 Tesla (TR:2.2s) was used to study 17 bipolar depressed inpts before and after repeated TSD treatment. The cognitive activation paradigm based on a go/no-go task was used. Words with a positive or negative moral value

(*e.g.*, brave/vile) were randomly presented in 4 sessions. Pts either responded to positive targets and ignored negative distractor or in an opposite manner. Using SPM2 with WFU_PickAtlas toolbox, we modeled 3 aspects of brain activation at each voxel in right DLPFC (BA 9): (1) contrast between response to positive or negative stimuli and resting condition, (2) contrast between response to positive and to negative stimuli, (3) regression between mood levels (factor), and response to positive or negative stimuli.

Results: BA9 baseline activity was not correlated with mood level. Response to treatment was paralleled by an increased neural response to emotional stimuli. Activity was correlated with perceived mood: happiness (visual analogue scale or VAS > 50) higher activity for negative and lower for positive stimuli; sadness (VAS < 50) lower activity for negative and higher for positive stimuli.

Conclusions: Response to total sleep deprivation treatment was paralleled by an increase in the role of right DLPFC in the management of emotion, with specific effects when processing emotionally tuned stimuli.

Keywords Bipolar disorder, Total sleep deprivation, BOLD fMRI

NEURAL CORRELATES OF ANTIDEPRESSANT RESPONSE TO SLEEP DEPRIVATION: A 3.0T BOLD FMRI EVENT-RELATED STUDY OF MORAL THINKING

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Objectives: Total sleep deprivation (TSD) causes rapid antidepressant effects in pts affected by a major depressive episode. PET studies show that recovery from depression is paralleled by reduction in subgenual cingulate and increase in right prefrontal cortex CBF, with responders to TSD showing higher metabolism in ventral anterior cingulate at baseline, and greater decreases after treatment. We explored the following hypotheses: 1) the cognitive activation linked to moral thinking results in activation of the above described ROIs, and 2) antidepressant TSD changes it.

Methods: Gradient echo echo-planar imaging at 3.0 Tesla (TR:2.2s) was used to study 12 bipolar depressed inpts before and after repeated TSD treatment. The cognitive activation paradigm consisted of a go/

no-go task. Words with a positive or negative moral value (*e.g.*, brave/vile) were randomly presented in 4 sessions. Pts either responded to positive targets and ignored negative distractors or they responded in an opposite manner. Using SPM2 ANOVA with wfu pickatlas toolbox, we modeled 2 aspects of brain activation at each voxel: (1) contrast between response to positive or negative stimuli and resting condition and (2) contrast between response to positive and negative stimuli. This latter contrast was expected to elicit the neural correlate of the processing of morally tuned information.

Results: Baseline maximal BOLD significant signal changes with the positive-negative contrast were observed in 2 clusters: left anterior cingulate and bilateral posterior cingulate. After TSD treatment, maximal significant changes in BOLD signal with the positive-negative contrast were observed in a cluster located in the dorsolateral prefrontal cortex bilaterally. Anterior cingulate activation persisted, while no significant activation was detected in the posterior cingulate. After TSD treatment a significant cluster of activation appeared within the insula as well.

Conclusions: BOLD signal changes linked with the processing of morally tuned stimuli were observed in the expected ROIs. The same stimuli activated different neural networks at baseline and after treatment. Recovery from bipolar depression was paralleled by a decrease in the role of the cingulate in the processing of morally tuned stimuli and an increase in the role of DLPFC.

Keywords Bipolar disorder, Total sleep deprivation, BOLD fMRI, Neural correlates of cognitive activation

THE LACK OF TIME-DEPENDENT EFFECT OF MELATONIN ON RADIATION–INDUCED APOPTOSIS IN CULTURED RAT LYMPHOCYTES

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Objectives: We investigated the effect of melatonin on apoptosis in cultured lymphocytes after total body (TB) or abdominopelvic (AP) ionizing

radiation (8 Gy) of rats either in the morning (1 hour after lights on: HALO) or evening (13 HALO).

Methods: 69 adult male rats wee synchronized to 12 h a L:D cycle (lights on 08:00 h) and randomized to 10 groups as shown in the table.

Group	Time of sacrifice	TB Irradiation	AP Irradiation	Ethanol (20%)	Melatonin Treatment
1A (Control)	1 HALO	−	−	−	−
−1B (Control)	13 HALO	−	−	−	−
−2A	1 HALO	+	−	+	−
−2B	13 HALO	+	−	+	−
−3A	1 HALO	−	+	+	−
−3B	13 HALO	−	+	+	−
−4A	1 HALO	+	−	−	+
+4B	13 HALO	+	−	−	+
+5A	1 HALO	−	+	−	+
+5B	13 HALO	−	+	−	+

Melatonin or vehicle (ethanol) was administered to the rats immediately before, after, and 2 h preceding TB or AP irradiation (melatonin doses were: 10 mg/kg, 20 mg/kg, and 100 mg/kg, i.p., respectively). 48 h after irradiation, all animals were sacrificed using ketamine. The lymphocytes were isolated and cultured. The different stages of apoptosis and DNA fragmentation were evaluated for quantitative and qualitative analysis, respectively. The interaction between time and treatment and differences between groups were statistically analyzed by two-way ANOVA.

Results: Radiation induced the rate of apoptosis in rat lymphocytes after both TB (groups 2A and 2B) and AP (groups 3A and 3B) irradiation, irrespective of its time of administration. Melatonin treatment did not influence the rate of apoptosis at either treatment time. There was no significant interaction between time and treatment.

Conclusions: Previous studies have shown that *in vitro* melatonin administration decreases the percentage of apoptotic cells at higher, but not in lower, doses in cell culture. It has also been reported that 100 mg/kg melatonin *in vivo* decreases radiation–induced DNA strand breakage and lipid peroxidation in rat brain. Therefore, the effect of melatonin on apoptosis seems to depend on the dose and cell type, but not application time.

Keywords Melatonin, Ionizing radiation, Lymphocytes, Apoptosis, Circadian treatment time

INDEPENDENT PROGNOSTIC VALUE OF THE REST-ACTIVITY CIRCADIAN RHYTHM (RAR) ON OVERALL SURVIVAL (OS) IN PATIENTS (PTS) WITH METASTATIC COLORECTAL CANCER (MCC) RECEIVING FIRST LINE CHEMOTHERAPY WITH 5-FLUOROURACIL, LEUCOVORIN AND OXALIPLATIN: A COMPANION STUDY TO EORTC 05963

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Background: In tumor bearing rodents, disruption of the RAR results in faster tumor growth and a shorter OS (*Cancer Res.* 64:7879; *JNCI* 94:690; *Cell* 111:41). In single institution prospective trials on pts with metastatic breast cancer (*JNCI* 92:994) and MCC (*Clin. Cancer Res.* 6:3038), a disrupted RAR also resulted in a shorter OS. In this multicenter prospective trial, we studied the prognostic value of the RAR in MCC patients receiving first line FOLFOX-2 *vs.* 4-day chronomodulated infusion of the same drugs.

Methods: To assess the RAR, 130 pts out of a total of 564 pts in 9 centers wore a wrist actigraph to record activity (the number of wrist movements per min) for 3 days before starting therapy. Two RAR parameters, validated in previous clinical studies, were derived: the autocorrelation coefficient at 24 h (r24), indicating the robustness of the activity pattern over the 24 h period, and the dichotomy index ($I < O$), quantifying the differences in activity distribution between the rest and active phases. The prognostic values of r24 and $I < O$ were assessed by the logistic regression model for response and toxicity and by the Cox Proportional Hazard model for OS.

Results: RAR parameters were not predictive for response or toxicity. When RAR parameters were categorized into quartiles, r24 and $I < O$ were detected to be potential prognostic factors for OS in a univariate analysis. The relative risk of death was 1.31-fold greater in the lowest *vs.* highest $I < O$ quartile. In the Cox Proportional Hazard model, $I < O$ was confirmed as an independent predictor of OS ($p < 0.0003$), along with CEA ≥ 50 ng/mL ($p = < 0.0001$), WHO PS 1 or 2 ($p = 0.0129$) and number of metastatic sites ≥ 2 ($p = 0.039$). Using the bootstrap

technique, I < O had a 70% probability of inclusion in the Cox model, making it one of the strongest prognostic factors for OS.

Conclusions: This multicenter prospective study establishes the strong and independent prognostic value of the rest-activity circadian rhythm for the overall survival of pts diagnosed with metastatic colorectal cancer.

Keywords Metastatic colorectal cancer, Rest-activity circadian rhythm, circadian rhythm predictors of cancer survival, Wrist actigraphy

QUALITY OF LIFE (QOL) CORRELATES WITH THE REST-ACTIVITY CIRCADIAN RHYTHM (RAR) IN PATIENTS (PTS) WITH METASTATIC COLORECTAL CANCER (MCC) ON FIRST LINE CHEMOTHERAPY WITH 5-FLUOROURACIL, LEUCOVORIN AND OXALIPLATIN: AN INTERNATIONAL MULTICENTER STUDY (EORTC 05963)

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Background: The QoL assessment by questionnaires is limited by their subjective nature. Actigraphy monitoring is non-invasive and provides quantitative parameters of the RAR that correlate with QoL items and can complement QoL questionnaires (*Clin. Cancer Res.* 2000: 6; 3038). In a multicenter trial, we prospectively investigated the correlation between QoL and RAR parameters in previously untreated MCC pts.

Methods: Before the first chemotherapy course, 94 MCC pts completed the EORTC QLQ-C30 and had RAR wrist actigraphy assessment for 3 days by a small wrist-watch sized device, actigraph, to records the number of arm movements per min. Three validated RAR parameters were calculated: mean activity (mAct), autocorrelation coefficient at 24 h (r24, indicating the robustness of the activity pattern over the 24 h period), and dichotomy index (I < O, quantifying the differences in activity distribution between the rest and active phase). QoL items were correlated with RAR parameters by the Spearman rank 2-sided test.

Results: Global QoL correlated positively with mAct ($r = 0.36$; $p = 0.0005$), r24 ($r = 0.35$; $p = 0.0006$) and I < O ($r = 0.40$; $p < 0.0001$).

I < O was negatively correlated with fatigue ($r = -0.39$; $p < 0.0001$), appetite loss ($r = -0.38$, $p < 0.0001$), and pain ($r = -0.37$; $p = 0.0002$). A positive correlation was found between r24 and social functioning ($r = 0.35$; $p = 0.0006$) as well as I < O and physical functioning ($r = 0.36$, $p = 0.0004$). Conversely, RAR parameters were poorly or not correlated with insomnia and cognitive and emotional functioning.

Conclusions: This prospective multicenter study has established the correlation between the RAR and global QoL, several symptom scores, and functioning scales in chemotherapy-naïve MCC pts. The circadian timing system, that generates the RAR, can be disrupted in cancer. Monitoring this system gives an objective and quantitative estimate of QoL and symptom clusters in cancer pts. The strength of the relation between RAR output and QoL is being further assessed during interventions targeted at the circadian system.

Keywords Metastatic colorectal cancer, Wrist actigraphy, Sleep-activity, Circadian rhythm, Quality of life

GENDER AS A PREDICTOR FOR OPTIMAL SCHEDULE OF OXALIPLATIN, 5-FLUOROURACIL AND LEUCOVORIN IN PATIENTS (PTS) WITH METASTATIC COLORECTAL CANCER (MCC): RESULTS FROM EORTC RANDOMIZED PHASE III TRIAL 05963

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Background: Gender-related differences characterize colorectal cancer incidence, gene expression patterns, as well as 5-fluorouracil (5-FU) pharmacology (dihydropyrimidine dehydrogenase activity, 5-FU clearance, and circadian rhythm amplitude, which is lower in females (F) than males (M)). Yet gender is not a prognostic factor for overall survival (OS) in MCC. Here, the impact of gender was examined in a randomized multicenter trial in 564 MCC pts where no difference in OS was found between two schedules of oxaliplatin, 5-FU and leucovorin: FOLFOX or 4-day chronomodulation (CHRONO) (Giacchetti et al., ASCO 2004, #3526)

Methods: The prognostic and predictive value of gender for effect of CHRONO on OS was tested using the log-rank test and the Cox

proportional hazards regression model. Analyses further assessed gender prognostic and predictive values for Progression Free Survival (PFS), Objective Response (OR), and Overall Toxicity (OTOX: occurrence of any grade of 3 to 4 main toxicity per pt).

Results: Gender was not a prognostic factor for OS ($p = 0.11$), PFS, or OR. F pts displayed significantly higher OTOX than M (72.6% *vs.* 55.9%; $p < 0.001$). Strong gender-schedule interactions were demonstrated for both PFS ($p = 0.008$) and OS ($p = 0.001$). In F, the median OS was 19.3 months (mo) on FOLFOX as compared to 16.3 mo on CHRONO (Log Rank, $p = 0.027$, 38% larger risk of death). Conversely, the median survival of M pts was improved by 3.1 mo with CHRONO as compared to FOLFOX (21.4 *vs.* 18.3 mo; $p = 0.018$, 25% lower risk of death). The gender-schedule interaction was present in each age and performance status subgroup. The predictive value of gender for schedule effect on OS was confirmed following adjustment for pt characteristics.

Conclusions: Gender strongly and significantly predicted for the best dynamic scheduling of chemotherapy to optimize OS. The CHRONO schedule tested here produced a clear survival advantage over FOLFOX in M (2/3 of the MCC pts). Gender-based differences in circadian pharmacology will guide the development of a better circadian delivery schedule in F MCC pts.

Keywords Metastatic colorectal cancer, Gender-dependent differences in response to cancer treatment, Cancer survival

DIFFERENCES IN DAY/NIGHT BLOOD PRESSURE (BP) RATIO BETWEEN NORMAL PREGNANCY, GESTATIONAL HYPERTENSION, AND PREECLAMPSIA

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Objectives: Several authors have found a reduced drop in BP by night in preeclamptic women. The diminished nocturnal decline in BP has been also associated with an increase in end-organ damage and cardiovascular events. Accordingly, we have compared the day/night ratio between normotensive and hypertensive pregnant women.

Methods: We analyzed 2523 BP series obtained from 245 normotensive pregnant and 189 women who developed gestational hypertension

or preeclampsia. BP was measured at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night for 48 h every 4 wks from the first obstetric visit until delivery. The day/night ratio was obtained for each BP profile as the% decline in the nocturnal relative to diurnal BP mean, taking into account the actual rest-activity cycle of each woman determined by diary and physical activity sampled at 1 min intervals by wrist actigraph.

Results: Statistically significant differences were documented in the day/night BP ratio between healthy and complicated pregnancies at all gestational ages ($p < 0.001$). In the first trimester, women with a final diagnosis of preeclampsia showed the higher average day/night ratio among the 3 groups of women investigated. The ratio was unchanged between the first and second trimesters for normotensive women and those who developed gestational hypertension, while it started to decrease in women who developed preeclampsia. In the third trimester, women with preeclampsia showed the smallest average ratio, and women who developed gestational hypertension the highest, markedly above the average ratio of normotensive women ($p < 0.001$).

Conclusions: This study on women systematically studied by ABPM along gestation indicates that preeclampsia is characterized by a significantly different pattern of change in day/night ratio along gestation compared to normotensive pregnancies or women who develop gestational hypertension without proteinuria.

Keywords Day/night Blood pressure ratio, Gestational hypertension, Preeclampsia, Blood pressure circadian rhythm

LEUKOCYTE COUNT AND GLOBULAR SEDIMENTATION VELOCITY ARE INCREASED IN SUBJECTS WITH METABOLIC SYNDROME (MS)

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Objectives: Previous studies have established a marked association between MS and increased cardiovascular risk. Moreover, proinflammatory and prothrombotic states have also been identified as major components of MS. We have investigated if some readily available and cost-effective markers of inflammation may be associated with MS.

Methods: We studied 1709 subjects (876 men), 48.2 ± 13.8 (mean ± SD) yrs of age; 499 were normotensive (according to ambulatory blood pressure [BP] criteria) and 1210 were hypertensive. All subjects were untreated at the time of study. BP was measured for 48 h, at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night.

Results: MS was present in 576 of the subjects (33.7%). Subjects with MS were characterized by a significant increase in leukocyte count (7170 *vs.* 6613 cells/μL; $p < 0.001$), neutrophils (4161 *vs.* 3825 cells/μL; $p < 0.001$), lymphocytes (2247 *vs.* 2076 cells/μL; p 0.001), and globular sedimentation (15.0 *vs.*11.6 mm; $p < 0.001$) compared to subjects without MS. Regarding ambulatory BP, the largest difference between groups was found for the nocturnal BP mean and the day/night BP ratio, indicating a tendency towards a more non-dipper profile in MS. Globular sedimentation was significantly correlated with the decrease in the day/night ratio of both systolic and diastolic BP ($p < 0.007$).

Conclusions: Results document a significant increase in inexpensive markers of inflammation and atherosclerosis in MS. Leukocyte count and globular sedimentation have previous been linked to coronary heart disease. These markers are also highly correlated with a blunted nocturnal decline in BP, which is more prevalent in MS.

Keywords Metabolic syndrome, globular sedimentation, leukocyte count, Blood pressure circadian rhythm, blood pressure ratio

DIFFERENCES IN THE SEASONAL VARIATION OF PLASMA GLUCOSE BETWEEN DIPPER (DH) AND NON-DIPPER (NDH) HYPERTENSIVE PATIENTS (PTS)

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Objectives: NDH pts (<10% decline in the nocturnal relative to the diurnal blood pressure (BP) mean) are more glucose intolerant than DH pts. The lack of nocturnal decline in BP seems to be associated with an increase in cardiovascular events, which are most prominent during the winter. Accordingly, we quantified and compared a possible yearly variation of plasma glucose in DH and NDH pts.

Methods: We studied 2,443 pts with grade 1-2 hypertension (1,193 men), 52.7 ± 13.6 (mean ± SD) yrs of age. BP was measured for 48 h at

20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night. The circannual variation of plasma glucose was established for all pts and for subgroups of DH ($n = 1{,}216$) and NDH ($n = 1{,}227$) by multiple-component analysis.

Results: Plasma glucose was characterized by a significant seasonal variation ($p = 0.009$) with a mean value of 105 mg/dL, double circannual amplitude of 8 mg/dL, and time of peak value in March, with a secondary peak on September. A similar pattern of seasonal variation also characterized DH and NDH pts analyzed as separate groups. Throughout the year, NDH pts showed significantly higher plasma glucose levels than the DH pts (difference in annual mean of 7.5 mg/dL; $p < 0.001$).

Conclusions: The circannual variation in glucose is correlated in time with the reported annual variation in coronary events, as well as with the seasonal variation of fibrinogen, an independent marker of myocardial infarction and stroke (*Circulation*, 2003;108:1101–1106). The elevation of plasma glucose in ND compared to DH pts supports the documented tendency of non-dipping among diabetic hypertensive pts. Results also support the association between ND circadian BP pattern and increased risk of cardiovascular events, which are more prominent during the late winter months.

Keywords Glucose, Circannual rhythm, Blood pressure, Non-dipper hypertension, Dipper hypertension

COMPARATIVE PREVALENCE OF NON-DIPPER (ND) BLOOD PRESSURE (BP) PATTERN IN PATIENTS (PTS) WITH AND WITHOUT OBSTRUCTIVE SLEEP APNEA (OSA)

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Objectives: There is still considerable debate regarding the relationship between OSA and hypertension, as well as between OSA and the lack of the nocturnal decline (ND) on BP. Accordingly, we quantified the prevalence of hypertension and ND BP pattern in pts with OSA.

Methods: We studied 378 OSA pts (315 men), 54.6 ± 10.3 (mean $\pm$ SD) yrs of age, whose diagnosis was corroborated by overnight polysomnography. BP was measured for 48 h, at 20 min intervals from

07:00 to 23:00 h and at 30 min intervals over night. Diurnal and nocturnal BP means were calculated for each pt according to the individual sleep-wake schedule determined by wrist actigraphy and used to classify each pt as a dipper (D) or ND (nocturnal BP decline <10%). Hypertension was established on the basis of elevated diurnal, nocturnal, or 24 h mean of systolic or diastolic BP (*Am. J. Hypertens.* 1993;6:201S-206S). The incidence of ND was compared with that of a reference sample of 1184 pts with grade 1-2 essential hypertension (512 men), 52.1 ± 13.9 yrs of age, without OSA and who followed the same protocol of BP and actigraphy monitoring

Results: Results indicate a very high 77.8% prevalence of hypertension in OSA pts, although only 51% were under treatment with antihypertensive medications. The prevalence of ND pattern was 44.9% among untreated pts, and even higher, 61.3%, among treated pts. For non-OSA hypertensive pts, the prevalence of ND was not statistically smaller; it was 40.4% among untreated and 56.6% among treated hypertensive pts.

Conclusions: This trial indicates a very high incidence of hypertension in OSA patients. The prevalence of ND, however, is comparable with that found for hypertensive patients without OSA.

Keywords ABPM, Obstructive sleep apnea, Non-dipper blood pressure pattern, Circadian rhythm

BIOLOGICAL MARKERS OF THE DAY/NIGHT BLOOD PRESSURE RATIO (DNR) DETERMINED BY AMBULATORY MONITORING IN HYPERTENSIVE PATIENTS (PTS)

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Objectives: Lack of nocturnal decline in blood pressure (BP) has been associated with an increase in cardiovascular risk and events. The mechanism underlying the diminished nocturnal BP decline is still unclear, although recent reports reveal a high prevalence of non-dippers in the elderly, diabetics, and renal pathology. We investigated potential markers of the DNR, calculated as the percent decline in the nocturnal relative to the diurnal BP mean, in hypertensive pts.

Methods: We studied 1666 pts with grade 1 to 2 essential hypertension (784 men), 52.6 ± 13.6 (mean ± SD) yrs of age. BP was measured for 48 h

at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night. A model for the DNR of systolic and diastolic BP was obtained by multiple regression analysis, using as potential predictors variables obtained from blood and urine tests, as well as demographic parameters, including age, height, weight, body mass index (BMI), waist and hip perimeters, and conventional BP values.

Results: Age, glucose, waist perimeter, fibrinogen, plasma creatinine, and BMI showed, in this order and as individualized parameters, the strongest (negative in all cases) correlation with the DNR of both systolic and diastolic BP. Conventional BP values a nd gender were not significantly correlated with the DNR. Multiple regression analysis indicated that the best model for prediction of DNR included age, waist perimeter, glucose, fibrinogen, and either creatinine or albumin/creatinine ratio. The predictive value was significantly increased when the 24 h mean of pulse pressure was also included in the model.

Conclusions: We found a strong association of a reduced nocturnal decline in BP (non-dipping) with aging, central obesity, progression to diabetes (glucose), progression to renal damage, and markers of cardiovascular risk (fibrinogen and pulse pressure).

Keywords Day/night blood pressure ratio, Glucose, Fibrinogen, Circadian rhythm, Hypertension

PREDICTABLE CHANGES IN THE DAY/NIGHT BLOOD PRESSURE RATIO (DNR) WITH AGING IN UNTREATED HYPERTENSIVE PATIENTS (PTS)

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Objectives: Non-dipping blood pressure (BP) pattern increases the risk of cardiovascular and cerebrovascular events, nephrosclerosis, and progression to end-stage kidney failure in renal patients. Some studies have documented a reduced BP DNR in elderly pts. Accordingly, we have investigated the changes in the BP DNR as a function of age in untreated hypertensive pts.

Methods: We studied 1553 untreated grade 1 to 2 essential hypertension pts (751 men), 49.5 $\pm$ 13.5 (mean $\pm$ SD) yrs of age, range 18 to 92 yrs.

BP was measured for 48 h at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night.

Results: The DNR of systolic BP was unchanged until 50 yrs of age (11.8 ± 0.2), and then decreased very rapidly at a rate of 0.39/yr ($p < 0.001$). The DNR of systolic BP decreased from 11.2 to 4.5 when age increased from 55 to > 70 yrs. For diastolic BP, the DNR first decreased at age 40 (18.3 ± 0.3 for pts < 40 yrs of age), was stable until age 55 (15.9 ± 0.3), and then decreased at a rate of 0.31/yr, reaching the lowest value of 10.6 in pts >70 yrs of age. The prevalence of non-dipping BP pattern significantly increased from 36.3% in pts <40 yrs to 71.2% in pts >70 yrs of age ($p < 0.001$).

Conclusions: The DNR BP ratio in untreated hypertension is stable until 55 yrs and then decreases vary rapidly with increasing age. The blunted nocturnal decline of BP with aging and corresponding increasing prevalence of non-dipping BP pattern should be taken into account to establish a proper chronotherapuetic strategy for the treatment of elderly hypertensive pts.

Keywords Non-dipper blood pressure pattern, 24 h ABPM, Aging, Day-night blood pressure ratio, Hypertension

SYMPOSIUM III-CHRONOBIOLOGY OF NUTRITION, APPETITE CONTROL, AND OBESITY

CHRONONEUROBIOLOGY OF APPETITE AND WEIGHT HOMEOSTASIS

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Feeding in vertebrates is a periodic behavior. Information amassed during the past decade has shown that the hypothalamus integrates metabolic, neural, and hormonal signals to evoke the intermittent appetitive drive for daily management of energy homeostasis. The two facets of energy homeostasis, energy intake and expenditure, are regulated by two distinct but interconnected networks in the hypothalamus. The appetite-regulating network propagates neuronal impulses for appetitive drive, and the energy expenditure network generates signals to expend energy appropriately on a min-to-min basis. The network of neuropeptide Y (NPY), a physiological appetite transducer, and cohorts is the conductor of the appetite regulating orchestra in the arcuate nucleus-paraventricular

nucleus (ARC-PVN) of the hypothalamus. The circadian and ultradian rhythmic secretions of NPY produce the daily episodic feeding patterns. Increased NPY release initiates appetitive drive directly and simultaneously represses the anorexigenic melanocortin signals in the ARC-PVN axis. In addition, reciprocal circadian and ultradian rhythmicities in afferent hormonal signals, namely anorexigenic leptin from adipocytes and orexigenic ghrelin from stomach, enforce corresponding rhythmic patterns of NPY release in the ARC-PVN axis. This intricate temporal crosstalk among rhythmic peripheral cues and the hypothalamic NPY network are necessary for sustenance of weight homeostasis. Derangements in this interplay caused either externally by varied quality and timing of food intake and energy disposal, or internally by shifts in hormonal metabolic and genetic factors, or by mechanical/neurochemical insults, disrupt the homeostatic signal relay in the hypothalamus and result in a positive energy balance, progressive weight gain, obesity, and associated metabolic diseases. This new understanding of the chrononeurobiology of appetite has identified new targets for designing pharmacologic and gene transfer therapies to curb the current worldwide epidemic of obesity.

Keywords Neuropeptide Y, Leptin, Ghrelin, Obesity, Gene therapy, Appetite regulation, Eating behavior

TIMING OF EXERCISE WITH RESPECT TO MEALS AFFECTS CIRCADIAN GLUCOREGULATION

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Objectives: To measure changes in blood glucose, insulin, and the counter-regulatory hormones glucagon, growth hormone, cortisol, and catecholamines during the 24 h period with (1) exercise in fasted or post-absorptive state, and (2) exercise in post-prandial state.

Methods: Nine postmenopausal women (2 Afro-American, 7 Caucasian), 58.8 yrs old, BMI 26.7 kg/m^2, were studied in both trials. Equicaloric meals were provided at 10:00 and 17:00 h. Treadmill exercise was at 40% of maximal effort. Exercise before the meals (EXBM) occurred between 07:00 and 11:00 h and 14:00 and 16:00 h. Exercise after the meals (EXAM) was between 11:00 and 13:00 h and 18:00 and 20:00 h.

Results: Approximately 400 Kcal were expended during each daily bout of exercise. Subjects' weight was unchanged between the two trials

(74.43 ± 4.3 kg EXBM *vs.* 73.72 ± 4.5 kg EXAM). In EXBM, post-absorptive glucose concentrations declined to 3.75 mM after the AM exercise and to 4 mM after the PM exercise and remained at that level throughout the night. By contrast in EXAM, post-absorptive glucose concentration was maintained at about 5 mM throughout the 24 h. The 18% reduction in plasma glucose in EXBM relative to EXAM is most likely due to 50% greater post-prandial insulin response, and inadequate counter-regulatory hormone compensation.

Conclusion: Exercise before meals reduces circadian plasma glucose more than exercise after the meals.

Keywords Time of exercise, Glucoregulation, Glucose, Meal timing

RAMADAN FASTING AND THE CIRCADIAN SYSTEM

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One of the most important rules of Islam is that any healthy adult Muslim must refrain from eating, drinking, smoking, and sexual relations from sunrise to sunset during the month of Ramadan, the ninth month of the Muslim calendar. Since this is a lunar calendar, the timing of this month of fast changes each year and the duration of restricted food and beverage intake can vary from between 12 to 16 h. Intake is restricted to the night hours within a short span of time, which thus delays sleep and reduces its duration. Environmental factors, such as the timing of the rest-activity cycle and meals, play a part in the synchronization of individuals to the 24 h day and are accordingly known as synchronizers. They modulate or modify one or several of the parameters characterizing the circadian rhythm of biologic variables. We reported the alteration with Ramadan of the circadian patterns of plasma gastrin, insulin, glucose, calcium, and gastric pH in both healthy and healed duodenal ulcer patients, and strikingly the presence of a number of these alterations one month after the end of Ramadan which enforces the relevance to the debate about the strength of meal-time as a rhythm synchronizer in humans. We observed different kinds of modification during Ramadan for variables considered as markers of the circadian system: melatonin maintained its circadian rhythmicity, but with decreased amplitude and a trend towards a phase delay, and cortisol maintained its circadian rhythmicity, but with a biphasic pattern. Our findings indicate that these rhythms may be controlled by

different oscillatory components, as other authors have suggested. Indeed the hormones under study expressed different circadian sensitivities to the altered synchronizers which support the theory of a hierarchy of oscillators and individuality of oscillators as an alternative to a single master clock. Ramadan is in essence a seasonal external factor as it changes its annual location but besides the possible changes in the induced effects according to the duration of fasting time, one cannot rule out a superimposed seasonal variation of the sensitivity of the target rhythms (phase shifting, amplitude, mean level) to Ramadan.

Keywords Ramadan, Fasting, Rhythm desynchronization, Hormones, Melatonin

CHRONOBIOLOGIC FACTORS IN WEIGHT CONTROL AND OBESITY

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Obesity has reached epidemic proportions in developed countries like the USA and West Europe, and is becoming more of a problem in developing countries as well. In the USA, a shortening of the life expectancy is predicted due to obesity-related morbidity and mortality. The recent progress in the understanding of the afferent and efferent branch of the appetite regulating system, and the molecular and biochemical events in the central hypothalamic processing unit may within the near future open avenues for preventive and/or therapeutic intervention. Since the appetite regulating system is closely integrated with the human circadian time organization, such interventions will be most successful if placed at certain circadian stages which still will have to be better defined. Before specially targeted interventional therapy has become available, chronobiologic studies can provide some guidance for behavior modification for weight control and avoidance of obesity. Animal experiments have shown the differences in food utilization as a function of time of feeding. Irrespective of the composition of the food given over a restricted 4 h time span in mice, the greatest weight gain or least weight loss due to restricted feeding was observed when the food was given prior to the onset of the daily rest span in LD12 h:12 h synchronized animals. Experience with human feeding patterns in clinically healthy subjects and in pts with night eating syndrome indicates a similar behavior in human subjects. Similar to the results obtained in experimental animals, the best calorie

utilization in human subjects occurs after food uptake in the evening, prior to nocturnal rest. The reason for this is multifactorial with differences in intestinal handling of food and decreased energy expenditure after a meal ingested at nighttime. Another behavioral factor to be considered is sleep deprivation. Our electrified society sleeps, on average, several hours less than our agricultural forefathers. Added to this is, in 15 to 20% of our working population, sleep loss due to night and shift work. Sleep deprivation leads to suppression of growth hormone secretion with simultaneous rise of cortisol and insulin concentrations. In a chronic state, this leads to a relative growth hormone deficiency, hypercortisolism, and hyperinsulinism to which is added relative insulin resistance providing several of the features of "metabolic syndrome." The behavior change needed to counteract the tendency for weight gain may be relatively small and achievable without drastically altering current life style. In this respect, chronobiology may be helpful in contributing to body weight management. This should include avoidance of large and especially carbohydrate-rich meals prior to the daily rest span and avoidance of sleep deprivation and its metabolic consequences. Timing of drug treatment with centrally (e.g., transnasally) or generally administered agents is expected to play a role in the near future.

Keywords Obesity, Feeding patterns, Circadian rhythms, Sleep deprivation, Insulin, Growth hormone, cortisol, metabolic syndrome

PLENARY LECTURE

DAY/NIGHT RHYTHMS AS DETERMINANTS OF CARDIOVASCULAR HEALTH AND DISEASE

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Heart and blood vessel physiology and the occurrence of cardiovascular disease exhibit diurnal patterns. Molecular oscillators, originally identified in the central clock, have been identified recently in peripheral organs including the heart. We have begun to delineate diurnal alterations in the activity of the cardiovascular genome and proteome in order to elucidate how the normal and diseased heart responds to differing demands between night and day. We first examined the myocardial expression of 12,488 genes in the normal murine heart under diurnal conditions. Variation in genetic activity was considerable, 1634 genes (~13% of genes

analyzed) exhibited statistically significant changes in expression across the 24 h cycle. Some genes exhibited rhythmic expression, while others showed abrupt change at the light:dark transitions. Importantly, these genes mapped to key biological pathways, including cellular growth and remodeling, respiration, and signal pathways. We then examined diurnal gene expression in heart disease, using a model of pressure-overload cardiac hypertrophy induced by aortic banding in mice. Genes involved in cardiac responses, such as blood pressure homeostasis, myocyte hypertrophy, and tissue remodeling, exhibited significant cycling changes in the banded heart and hypertensive aorta as compared to sham. Physiologic implications were examined by circadian desynchronization. We used two approaches. First we disrupted rhythms through a 10 h:10 h L:D cycling in aortic-banded mice, and second by we used a model of prolonged rhythm disruption in τ mutant hamsters. We used multiple assessments of cardiovascular pathophysiology, including gravimetrics, histopathology, gene expression, behavioral analysis, biochemistry, and functional physiology. We showed that rhythmicity is integral to cardiovascular remodeling. Rhythm disturbance has significant adverse effects on the heart and blood vessels. We then hypothesized that the efficacy of drug therapy in cardiovascular disease may also exhibit diurnal rhythmicity. We investigated the efficacy of *i.p.* captopril (ACE inhibitor) on cardiac remodeling, in aortic-banded mice, when administered in the day vs. night. Captopril given when the mice normally slept (during the AM) significantly reduced ventricular mass, and improved cardiac function; captopril given when the mice were awake (during the PM) did not differ from placebo. These results indicate that molecular diurnal regulation is important for cardiovascular growth and remodeling. Changes do not occur uniformly over the day/night cycle, but are dynamic and temporal in nature. This is important for the study of cardiovascular disease, with implications for myocardial remodeling, timing of medications, and the development of novel therapeutic approaches.

Keywords Cardiovascular disease, Molecular oscillator genes, Circadian rhythm disturbances, Captopril, Chronopharmacology, cardiac remodeling

SYMPOSIUM IV-CHRONOBIOLOGY AND CHRONOTHERAPY OF HYPERTENSION

INTRODUCTION TO THE CHRONOBIOLOGY AND CHRONOTHERAPY OF HYPERTENSION

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Diastolic (D) and systolic (S) blood pressure (BP) exhibit 24 h variation. In uncomplicated essential hypertension, BP is lowest during normal nighttime sleep—by 10 to 20%—compared to the daytime mean level, rise sharply (by as much as 40 to 50 mm Hg in elderly persons) to peak or near peak level in the morning upon commencement of diurnal activity, and decline in the evening. In secondary hypertension, for example, due to renal disease or diabetes, the nocturnal decline in BP either is blunted or reversed, being higher during nocturnal sleep than daytime activity. In some pts, the nocturnal decline may be greater than normal, i.e., by more than 20% of the daytime mean, leading to inadequate blood perfusion to some organs, nocturnal syncope, nighttime falls, stroke, and optic nerve injury resulting in compromised eyesight. In addition, new data suggest the day-night pattern of BP in pts who are resistant to antihypertensive therapy is different from those who are responsive to such; the former display more of a 12 than 24 h waveform pattern of BP with peaks in the morning and early evening. Studies show it is the characteristics of the entire 24 h pattern in DBP and SBP and the total BP load per 24 h that are deterministic of the deleterious effects upon targeted end-organ tissues, in particular, the blood vessels and tissues of the kidney, eye, and brain, and the overall aging of the cardiovascular system. The extent of the morning rise in BP has been linked to increased risk of stroke, particularly in the morning, and it may also play a role in the etiology of myocardial infarct and sudden cardiac death at this time of day. The inadequate decline of BP during nocturnal sleep also has been linked to kidney injury, often accompanied by loss of albumin to urine. The treatment of hypertension generally neglects differences in therapeutic requirements between pts as dictated by the specific features of the BP 24 h pattern. Some US pharmaceutical companies now market medications that utilize special drug-delivery technology to better control of BP in the morning and daytime than conventional ones. A growing number of studies show the ingestion-time of BP-lowering medications can make an important difference on 24 h BP control. This chronopharmacology of antihypertensive medications must be taken into account to optimize the outcome of therapeutic interventions for hypertension to achieve a chronotherapy of conventionally formulated once-a-day agents. In this regard, the nighttime, as compared to daytime, dosing of certain calcium channel blocker, angiotensin converting enzyme inhibitors, and angiotensin receptor blockers has been shown to be more effective in controlling non-dipping nocturnal hypertension patterns. Moreover, Studies show great differences in the safety and therapeutic value of antihypertensive medications when ingested at different clock (biological) times.

The findings of the growing number studies that show prominent dosing-time-dependent differences in the effects of BP-lowering medications support the contention that antihypertensive agents be assessed at 2 or more different dosing times in drug registration and long-term intervention outcomes trials to determine administration-time-differences in effectiveness and safety.

Keywords Hypertension, Circadian rhythm, Chronopharmacology, Chronotherapy. End-organ injury, Blood pressure dipper pattern

CHRONOBIOLOGICAL APPROACH TO THE DIAGNOSIS AND TREATMENT OF ARTERIAL HYPERTENSION AND ACUTE CARDIOVASCULAR EVENTS

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The wide use of ambulatory blood pressure (BP) monitoring has led to the appreciation of the circadian structure of BP variability and its clinical relevance. It is now clear that the circadian pattern of BP results from reciprocal interactions between external stimuli synchronized to the rest-activity cycle (physical and mental activity, stress) and neurohumoral influences with an endogenous rhythmicity. The monoaminergic systems appear to integrate the major driving factors of temporal variability, but evidence also indicates a role of the hypothalamic-pituitary-adrenal, opioid, renin-angiotensin-aldosterone, and endothelial systems, as well as other vasoactive peptides The sleep-wake cycle, itself, results from the alternant prevalence of mutually inhibitory interactions between activating and deactivating systems and substances with established actions also on the cardiovascular system. Accordingly, a physiological temporal balance exists between the neurohumoral patterns of BP control. This balance is preserved also in uncomplicated essential hypertension; whereas, it is frequently lost in complicated and secondary forms of hypertension where gross alterations are found in the circadian profile of BP. These notions may contribute to a better understanding of the pathophysiology and therapeutics of changes in BP. Ambulatory BP monitoring may play a key role in this respect, especially if improvements in its technology and methodology will ensure a more accurate determination and analysis of BP profiles and variability.

Keywords Hypertension, Circadian rhythm, Blood pressure

PHYSIOPATOLOGIC IMPLICATIONS OF THE CIRCADIAN VARIABILITY IN BLOOD PRESSURE (BP) CHRONOTHERAPY WITH ANTIHYPERTENSIVE DRUGS

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BP displays appreciable predictable-in-time circadian variation. The chronotherapy of hypertension takes into account the clinically relevant features of the 24 h pattern of BP, *e.g.*, the accelerated morning rise in the morning and decline during nighttime sleep, plus potential administration (circadian) time determinants of the pharmacokinetics and dynamics of medications. Significant treatment-time differences in the kinetics (*i.e.*, chronokinetics) plus the beneficial and adverse effects (chronodynamics) of antihypertensive drugs are well known. Thus, evening, but not morning, dosing with isradipine significantly reduces and normalized nocturnal BP in hypertensive pts with chronic renal failure. Too, the dose-response curve, therapeutic coverage, and efficacy of doxazosin GITS are all markedly dependent on the circadian time of drug administration. The administration of nifedipine GITS at bedtime significantly reduces side effects, while increasing antihypertensive efficacy than administration upon awakening. Valsartan administration at bedtime, as opposed to upon wakening, results in improved diurnal/nocturnal BP ratio, such that the dosing time of valsartan can be chosen in relation to the dipper status of any given pt to improve therapeutic benefit and reduce cardiovascular risk. Moreover, in pts with resistant hypertension, chronotherapy has been shown to improve BP control and to reverse the non-dipper profile, which is highly prevalent among these pts. Nocturnal hypertension, which is characterized by the loss or even reversal of the expected 10 to 20% sleep-time BP decline, increases the risk of cardiac and cerebrovascular events. Chronotherapy constitutes a means of individualizing the treatment of hypertension according to the circadian profile of BP of each pt. The chronotherapeutic strategy represents a new option to optimize BP control and to reduce risk.

Keywords Chronotherapy, Hypertension, Blood pressure dipper pattern, Cardiovascular risk, Chronokinetics, Chronodynamics, Antihypertensive medications

SYMPOSIUM V-LIGHT THERAPY FOR PSYCHIATRIC DISORDERS: WHICH PATIENTS WILL BENEFIT?

MORE LIGHT AND MORE DARKNESS: A PLEA FOR GOOD ENTRAINMENT FOR MENTAL HEALTH

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One of the basic tenets of chronobiology is that temporal order is essential for health. In psychiatry, we are gathering more and more evidence that appropriate entrainment is a prerequisite for maintaining mental health, even if such evidence does not explain psychopathology. First, phase misalignments between the circadian clock and the sleep-wake cycle can cause decrements in mood in vulnerable subjects and initiate a depressive episode (the "dual" model of affective disorders). Conversely, shifting circadian or sleep phase can improve mood state. Thus, the stable timing of internal rhythms, with respect to the external light-dark cycle, appears to be important. Entrainment occurs primarily with the major zeitgeber, *i.e.*, light, first used as a treatment for winter depression (SAD). Light treatment has been extended to the treatment of non-seasonal depression, bulimia nervosa, and disturbed sleep-wake cycles such as in Alzheimer's dementia. Light can be combined with antidepressants or other chronobiologic treatments such as sleep deprivation or sleep phase advance. Remarkably, rapid cycling has been curtailed in pts maintained on long nights (*i.e.*, regular dark episodes). A similar regimen even appears to treat manic symptoms. Entrainment state is also influenced by psychopharmacologic agents. Antidepressants (particularly SSRIs) may stabilize entrainment by enhancing light sensitivity. In contrast, classic neuroleptics may induce circadian sleep-wake cycle disturbances rather than improve the nocturnal behavior they are intended to reduce. However, the atypical neuroleptics can improve entrainment, a characteristic that has been little researched so far. Schizophrenic pts manifest a variety of circadian rest-activity cycle disturbances; some may be related to the illness, some to the drugs used in their treatment, as above, and some to the changing patterns of light self-exposure which shifts the timing of sleep even more (in extreme cases, to free run). Thus, although understanding the role of adequate zeitgeber strength in psychiatric syndromes is still in its infancy, it promises to yield powerful and simple treatment strategies—more light and more darkness at the right times.

Keywords Entrainment, Sleep-wake cycles, Light therapy, Depression, Schizophrenia, Circadian rhythms

LIGHT TREATMENT OF BIPOLAR DISORDER

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An early hope of research on biological rhythms was that chronobiological interventions would become a benign alternative to more radical treatments of depression. The clinical usefulness of sleep-wake rhythm manipulations was however questioned by the short duration of their antidepressant effects. In the last 10 yrs, clinical research at our center has been concerned with discovering strategies to improve the usefulness of chronobiological treatments of bipolar depression. Combined treatments with lithium salts, low-dose antidepressant drugs, and chronobiological interventions, such as single or repeated total sleep deprivation (TSD), sleep phase advance (SPA), and morning light therapy (LT), allowed us to attain response rates of 60 to 70% and successfully prevent relapse, thus providing clinical psychiatrists with new instruments to achieve rapid and sustained antidepressant response in bipolar depressed pts. Synergistic effects were described between chronotherapeutic interventions, mood stabilizers, and both serotonergic and dopaminergic drugs. These effects were found to be influenced by the same clinical and demographic factors that have long been known to influence response to antidepressants (*e.g.*, history of drug-resistance, age, duration of episode, *etc.*). Taken together, these clinical observations suggest the possibility of common mechanisms of action, and/or of common individual factors affecting the antidepressant response to all these clinical interventions. Recent studies with functional neuroimaging techniques (PET, SPECT, BOLD fMRI) confirm that the antidepressant response to serotonergic, noradrenergic, and chronotherapeutic antidepressants is paralleled by similar changes in human brain function. In particular, we observed that the combination of TSD and LT in bipolar disorder could produce significant changes in the neural response to emotional stimuli, and in glutamate-glutamine cycle in specific brain regions. Recent data from genetic research helped us to partially explain the mechanism of action of chronobiological treatments. The influence of a functional polymorphism in the transcriptional control region upstream of the coding sequence of the 5-hydroxytryptamine transporter (5-HTTLPR) on response to TSD was found to be similar to that observed on response to serotonergic drug treatments. Moreover, patients treated with TSD followed by light treatment showed the same influence of 5-HTTLPR: the effects of light, wake therapy, and serotonergic drugs are influenced by the same individual somatic characteristic. Ongoing studies suggest that polymorphic genes

regulating the mammalian circadian rhythmicity (*e.g.*, CLOCK, hPER, GSK3-β) influence both core characteristics of bipolar illness and response to chronotherapy.

Keywords Bipolar depression, Light therapy, Antidepressant drug therapy, Total sleep deprivation, Functional polymorphism

LIGHT AND WAKE TREATMENT FOR NONSEASONAL DEPRESSION

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Objectives: Presenting data from a study using adjunctive bright light on non-seasonal major depression in a double-blind randomized clinical trial and highlighting a forthcoming study with light and wake therapy.

Method: Pts with non-seasonal major depression were blindly randomized to 5 wks of either 1 h bright light (BL) with 10.000 Lx or 0.5 h of dim red light (DL) with 50 Lx in the morning. All pts were treated with 50 mg sertraline/day. Pts were seen at weekly visits. Depression severity was assessed by use of the Hamilton Depression Rating Scale (17-item version), the Hamilton subscale (6 items), the Melancholia Scale (6 items), and the SIGHSAD (24 items).

Results: In total, 102 pts were studied. Mean age was 44.6 yrs; male/female ratio was 0.44. On all 4 outcome scales, the BL group had a statistically significantly faster reduction of depression scores. Response and remission rates were statistically significantly higher for the BL group. Thus, at week 5, the response rate (50% reduction on HAM-D_{17}) was 66.7% in the BL group compared to 40.7% in the DL group. The remission rates (a score of less than 8 on the HAM-D_{17}) were 41.7% in the BL group and 14.8% in the DL group. The level of blinding was acceptable as evaluated on a post-study self-assessment scale. Side effects were mild.

Conclusion: This study confirms the effects of adjunctive BL treatment in non-seasonal depression. The post-study evaluation implies a sufficient degree of blinding of light treatment conditions. A forthcoming study (Chronos) will use the results from this study applying long term BL treatment after initial wake therapy in bipolar depression as a means of sustaining the initial response to wake therapy.

Keywords Bright light therapy, Non-seasonal depression, Adjunct therapy of depression, Double-blind protocol, Sertraline

LIGHT AND ANTIDEPRESSANT TREATMENT OF SEASONAL AFFECTIVE DISORDER (SAD): SEARCH FOR A CIRCADIAN MECHANISM OF ACTION

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Objectives: Bright light (BL) therapy and antidepressant medications are effective treatments for SAD, or winter depression. The mechanism of action of BL therapy is unknown, but there are several major theories, including Lewy's circadian phase-shift hypothesis. A phase-shift hypothesis could explain the superiority of morning BL exposure and the relatively rapid therapeutic effects of light. Presumably, antidepressants are unlikely to work through a circadian phase-shift mechanism.

Methods: We recently conducted a multicenter, 8 wk randomized controlled trial of BL therapy (10,000 Lx white fluorescent light × 30 min in the early morning) *vs.* fluoxetine (20 mg/day) in SAD. As part of this trial, pts completed the self-report Morningness-Eveningness Questionnaire (MEQ) before and after treatment, and kept daily sleep diaries during the study. MEQ Scores can be used as a proxy for measurement of circadian phase in humans, as can sleep diary parameters such as sleep onset, sleep offset, and midpoint of sleep.

Results: Using these proxy measures of circadian phase, we were unable to demonstrate a significant correlation of changes in these measures with clinical response, or significant differences between light- and fluoxetine-treated pts.

Conclusions: Within the limitations of these data (*i.e.*, no physiological measures of circadian phase were done), our results are not consistent with a circadian phase-shift mechanism of the action for BL therapy or fluoxetine.

Keywords Seasonal affective disorder, Bright light therapy, Antidepressant medications, Circadian rhythms, Sleep

This study was funded by the Canadian Institutes of Health Research, CT62962. Light boxes were supplied by Uplift Technologies.

ORAL PRESENTATION SESSION E

NEW CLOCK GENES, *Erih1* AND *Erih2*

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Objectives: It is known that, as a master clock, the central controller of circadian rhythms is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. On the other hand, recently, peripheral clocks have been reported, that is, some kinds of cells have possess own clocks which may work independently from the SCN.

Results: We have been studying a certain peripheral gene using the yeast two hybrid technique to search for related proteins. We have found 2 new genes, which we named *hErih1* and *hErih2*. Moreover, we have found similar genes in the mouse, named *mErih1* and *mErih2*. Furthermore, we have found the expression pattern of these genes in various organs. Presently, we are investigating the function of these four genes using the RNA interference technique for knockdown of these genes and microarray analysis for examining down-related genes. We are also researching the protein structures of HERIH1, HERIH2, MERIH1, and MERIH2, and we are producing antibodies against HERIH1, HERIH2, MERIH1, and MERIH2 and the expression pattern of these proteins in various organs.

Conclusions: We are exploring a new gene family.

Keywords *hErih1*, *hErih2*, *mErih1*, *mErih2*, Peripheral clocks

$APC^{MIN/+}$ MICE HAVE NORMAL CIRCADIAN AND ESTROUS TIME STRUCTURES

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Objective: It is generally recognized that $APC^{min/+}$ (APC), compared to the wild type C57BL/6J (B6) mice, do not breed normally and have fewer pups per litter. Therefore, we asked whether the estrous cycles of female APC knockout mice are normal compared to B6 control mice. Because in mice, estrous and circadian cycles are linked, the FSH and LH bursts and the receptive estrous phase of the cycle occur during the nocturnal activity phase of the mouse. We also asked whether circadian activity/rest cycles of these mice are normal.

Methods: Ten week old APC ($n = 11$) and B6 ($n = 9$) mice were singly housed in regular cages with attached running wheels. The mice were

initially kept in 12 h light:dark (L:D) condition. On the 14th experimental day, the lighting regimen was shifted to constant dark (DD) and then to constant light (LL: 300 Lx) on the 27th day of the experiment. Body weights and vaginal smears were obtained daily. The circadian activity (number of running wheel revolutions/5 min) was recorded and analyzed using VitalView (Minimitter Co., USA). All mice were killed on the 33rd day of the experiment. Number of intestinal polyps, CBC, and spleen weight/body weight index were recorded. Circadian activity rhythm parameters were computed using Cosinor Analysis; comparisons (using ANOVA) and correlations were done using SAS 8.2.

Results: In 12L:12D condition, each mouse, of either strain, maintained statistically significant circadian rhythms in wheel running activity. The 24 h means and amplitudes of the wheel running rhythm of APC and B6 mice were identical. In DD, each mouse free ran with identical periods ($\tau = 23.5 \pm 0.15$ h) across strains. In LL, circadian activity rhythms broke down in each mouse, regardless of strain. In 12L:12D, the average number of estrous cycles completed in 18 consecutive days of observation was also identical across strain. No polyps were found in B6 mice, while there were 99.6 ± 10 polyps within the intestines of APC mice. In the APC mice, the greater the number of polyps, the fewer the number of receptive "estrus" phases ($r = -0.81$; $p < 0.01$).

Conclusions: Female APC knockout and B6 control mice have normal and similar circadian activity rhythms. Estrous cycling is also similar and normal. The sicker the APC mice (increased number of polyps), the fewer the receptive "estrus" cycle phases.

Keywords Circadian rhythm, Estrous cycle, Reproduction, APC mutation, Mice

STORES, TV, AND MOBILE-PHONE AT NIGHT RELATE TO M-E TYPOLOGY OF JAPANESE INFANTS

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Objectives: Our study examined the effects of usage of shops, TV or video, and mobile phone at night by Japanese parents on their morningness-eveningness (M-E) typology and their infants.

Methods: The Japanese version of the M-E questionnaire, originally constructed by Torsvall and Åkerstedt (1980), and another original

questionnaire including questions on factors relating to the '24 h society': *e.g.* "When you go to shops after sun-set, such as convenience store, restaurant for family-use, beer-garden, and renting-shop of video, dose your child accompany you?—(1) always, (2) occasionally, (3) sometimes, (4) rare, (5) never," were administered in June, 2004 to 740 children and their parents (638 mothers and 55 fathers) aged 0 to 6 yrs (they answered the two questionnaires both on themselves and their children) attending 1 of 9 municipal nursery schools in Kochi, Japan.

Results: The mean and S.E. of M-E scores of infants who always accompanied their parents to shops after sun-set was 19.45 ± 0.44 and more evening-typed than that of those who never accompanied their parents (21.16 ± 0.26) (Mann-Whitney U-test; $p < 0.001$). Parents who watched TV or video until after 23:00 h everyday were more evening-typed (16.71 ± 0.97) than those who never did such (19.25 ± 0.20) ($p < 0.01$). Parents who used mobile-phone mainly between 18:00 to 24:00 h were more evening-typed (18.89 ± 0.16) than those who mainly used between 12:00 to 18:00 h (19.99 ± 0.18) ($p < 0.001$). There was significant positive correlation between M-E scores of infants and their parents (Pearson-test: $r = 0.356$; $p < 0.001$).

Conclusions: Going shopping at night is directly associated with making Japanese infants evening-typed. Use of mobile-phones mainly at night and watching TV or video until 24:00 h by parents seems to have indirect effects on M-E scores of their infants.

Keywords Japanese infants, Japanese parents, Morningness-eveningness, Mobile-phone use, Television and video viewing

GENETIC VARIATIONS IN THE RHYTHMIC PHENOTYPE

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Introduction: The biological rhythm is a key tool which endows the organism with the means to adapt to its surrounding timing. Thus, the oscillatory phenotype of different individual organisms tends to be similar in a unique habitat.

Objectives: To reveal strain-dependent differences by monitoring the rhythmic patterns of enzymatic activity and to follow inheritance modes in the F1 generation.

Methods: 3 genotypic groups of 3-month-old male mice (BALB/c, c57BL/6J, and F1 generation) were exposed to 12L:12D illumination for 3 wks. Afterward, 3 to 4 mice of each group were sacrificed every 3 h over a period of 27 h. The kidneys were dissected and homogenized, and activities of alkaline phosphatase (AP) and creatine phosphokinase (CPK) were determined at each time point. Data fitness to an oscillatory pattern was assessed by 2-D table curve (SPSS, Inc. Cary, NC, USA) which enables one to compute basic sinusoid parameters (period, acrophase, amplitude, and mesor) as well as "general derived" features (area under curve, relative amplitudes and its change rate, sum of amplitudes, *etc.*).

Results: In most patterns (91.7%), the enzymatic pattern's phenotype displayed a compound rhythm, namely, having two distinct periodic components. Strain-dependent differences were demonstrated in most rhythms (75%) albeit the identical habitat, indicating the presence of measurable genetic polymorphisms. The inheritance of each parameter was individually controlled and most seemed to follow a simple recessive-dominant-codominant mode. However, new phenotypes were recognized especially among the "general-derived" features that manifested broader phenotypic variety (87% with new).

Conclusions: The rhythmic phenotype depends on the balance between the organisms' genotype and its efforts to adapt to the environment. Most patterns were compound, and each parameter was individually controlled by one or more genes. This combination endows the organism with the flexibility to achieve better optimization.

Keywords Biological rhythm, Genetic variations, Rhythm parameters, Entrainment, Inheritance

VARIATION IN RENAL CATALASE ACTIVITY INDUCED BY SODIUM NITROPRUSSIDE (SNP) IN MICE

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Objectives: Sodium nitroprusside ($Na_2[(CN)_5]$ FeNO) SNP is a vasodilating agent. It undergoes biotransformation *in vivo* to release free cyanide,

a very toxic metabolite. Catalase (CAT) is an enzyme present in several tissues, and plays an important role in hydrogen peroxide detoxification by metabolizing H_2O_2 into H_2O. The present study aims to investigate the effects of SNP dosing-time on CAT activity and its temporal variation in mice kidney.

Methods: Male *Swiss albino* mice weighing 25 to 30-g were purchased from the Central Animal House (SIPHAT, TN), acclimated to a temperature of 22 ± 2°C, and synchronized with a 12L:12D cycle for at least 3 wks. Animals were divided into 4 groups of 48 animals each (control I, control II, group I, and group II). Food and water were provided *ad libitum*. Mice of control I and group I were respectively treated once with a single *i.p.* injection of NaCl (0.9%) or SNP (2.5 mg/Kg) at 1 HALO (hours after light onset). Mice of two other groups (control II and group II) were similarly treated at 13 HALO. Animals were sacrificed at 8 different time-points (0, 1, 3, 6, 9, 12, 24, and 36 h) after SNP injection and the kidney was quickly excised and used to determine CAT activity (Sorbone, 1985). The Cosinor method was used for time series analysis and for estimation of circadian rhythm parameters (peak-time, mesor, and amplitude).

Results: A significant increase in CAT activity (5889 ± 350 µmol H_2O_2/g kidney) was observed 1 h after SNP injection in mice treated at 1 HALO compared to controls (4070 ± 132 µmol H_2O_2/g kidney) (Student-*t*-test; $p < 0.01$). At 13 HALO, the increase of CAT levels in treated groups (5167 ± 351 *vs.*2940 ± 434 µmoles H_2O_2/g kidney) was significant only 6 h after SNP injection. Both ultradian and circadian rhythms were detected by cosinor in all control and treated groups ($p < 0.05$); the prominent period was found to be $\tau = 24$ h. The peak-time (19 HALO) of the circadian rhythm was not modified in SNP-treated animals. The 24 h means were also unchanged in all treated groups (4082 ± 200 µmol H_2O_2/g kidney). Two-way ANOVA has shown that changes in CAT levels were statistically significant with regard to the circadian-time and drug injection. There was a significant interaction between drug and time-injection in animals treated at 1 HALO (ANOVA: $F_{5,21} = 4.4$; $p = 0.004$) and 13 HALO (ANOVA: $F_{5,26} = 12.8$; $p = 0.0001$).

Conclusions: SNP induces significant increase in CAT activity 1 and 6 h after injection in group I and group II, respectively. These differences are related to the increase of H_2O_2 levels produced by SNP. The CAT activity reached a high level to completely block the reduction of cell viability induced by SNP.

Keywords Catalase activity, Circadian rhythm, Mouse kidney, Sodium nitroprusside, Synchronization

INVESTIGATING THE LINK BETWEEN NIGHTTIME LIGHT POLLUTION AND BREAST CANCER: A GEOGRAPHIC INFORMATION SYSTEM (GIS)-ASSISTED ANALYSIS

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Objectives: To investigate the relationship between exposure to artificial illumination (light pollution) and breast cancer.

Methods: The analysis was conducted as two separate studies. During the first study, a satellite image of night light intensity in Israel was combined within a map of breast cancer rate, using GIS as a research tool. After the maps were overlapped, the means and standard deviations of night light intensity were calculated for localities with available cancer rates. A regression model was then used to identify factors affecting the rates of breast cancer. The second study included the *in-situ* measurements of night light intensity in four residential neighborhoods in Tel-Aviv, which were further analyzed in conjunction with local breast cancer rates.

Results: The study found a positive association between night light intensity and breast cancer rates, with a clear clustering of towns into groups with abnormally high, average, and low cancer rates. Regression analysis indicated more illuminated localities tend to exhibit (*ceteris paribus*) higher rates of breast cancer. The average income of the local population also had a positive association with breast cancer rates, as high-income households tended to use more artificial illumination in their homes. The second study of individual residential neighborhoods also revealed neighborhoods with high cancer rates were characterized by high night light intensity; whereas, in the neighborhoods with low cancer rates, night light intensity was significantly lower ($t = 5.114$; $p < 0.01$ for the high-income neighborhoods, and $t = 2.547$; $p < 0.05$ for low income neighborhoods).

Conclusions: The research findings reveal a clear association between exposure to nighttime artificial nighttime light and the incidence of breast cancer. According to the explanation we propose, intensive artificial light leads to the reduction of nighttime melatonin synthesis and secretion from the pineal gland. The intensive nighttime illumination is proposed to lead to a higher risk of breast cancer development, by causing changes in the photoperiod and affecting seasonal acclimatization of the immune system.

Keywords Light pollution, Breast cancer, Melatonin, Public health

INFLUENCE OF CYP2C9 GENOTYPE ON BLOOD PRESSURE (BP) PARAMETERS IN PATIENTS (PTS) WITH NOCTURNAL HYPERTENSION (NH)

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Objectives: BP varies as a diurnal rhythm. It is generally lower during the night than day time. Mean systolic blood pressure (SBP) greater than 125 mmHg during nighttime sleep denotes NH. Epoxyeicosatrienoic acids (EETs) are known to play an important role in vascular homeostasis, and therefore, may play a role in BP regulation. Cytochrome P450 (CYP) 2C9, a polymorphic enzyme involved also in the metabolism of many drugs—including some antihypertensives such as angiotensin converting enzyme inhibitors—are among the enzymes involved in the transformation of arachidonic acid to EETs. The aim of this study was to investigate the relationship between *CYP2C9* genotype and diurnal BP parameters in pts with NH.

Methods: Twelve pts with NH, diagnosed by 24 h ambulatory BP monitoring (ABPM), participated. Genomic DNA of pts was analyzed for the most common allelic variants of *CYP2C9* (*CYP2C9*2* and *CYP2C9*3*), by real time PCR methods.

Results: 5 subjects carried a detrimental allele, while 7 subjects could be classified as *CYP2C9*1/*1*. There was no significant difference between genotype groups for SBP parameters (24 h, daytime mean, nighttime mean, systolic BP load). However, almost all parameters for diastolic blood pressure (DBP) and heart rate (HR) (24 h mean, daytime mean, load), except nighttime mean, were lower in mutation carriers than in subjects homozygous for *CYP2C9*1* ($p < 0.05$).

Conclusions: Despite the small sample size, our findings suggest that the presence of *CYP2C9* detrimental alleles is associated with lower DBP and HR in pts with NH. Future studies on larger samples are needed to properly evaluate the impact of polymorphic CYP enzymes that are involved in the metabolism of vasoactive compounds and which are related to the pathophysiology of hypertension.

Keywords Pharmacogenetics, Diurnal rhythm, Nocturnal hypertension, ABPM, Blood pressure

GENETIC DISSECTION OF PSYCHOPATHOLOGICAL SYMPTOMS: INSOMNIA IN MOOD DISORDERS AND CLOCK GENE POLYMORPHISM

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Objectives: The Circadian Locomotor Output Cycles Kaput (CLOCK) gene is thought to be involved in the regulation of circadian rhythms, with its protein products driving transcription of other genetic components of the molecular oscillator. The possible role of 3111T/C CLOCK gene polymorphism in psychiatry is currently under investigation, given the high frequency of circadian rhythms disturbances in psychiatric illnesses, and the proposed role of chronobiological abnormalities in the pathogenesis of mood disorders. A polymorphism in the 3′ flanking region of the CLOCK gene has been shown to affect mRNA stability and half-life, with possible significant effects on the level of protein finally being translated. In mice, a mutation of CLOCK gene, called "C" variant, has been shown to lead to a lengthened circadian period and in healthy humans the CLOCK*C allele has been investigated as a predictor of "eveningness." Finally, in MD a significantly higher recurrence rate of illness episodes has been found in bipolar pts homozygous for the C variant.

Methods: In the present study, we investigated the possible effect of CLOCK variants on sleep disturbances in a sample of 620 subjects affected by Major Depressive Disorder (MDD) and Bipolar Disorder (BD). All pts were evaluated using the Operational Criteria for Psychotic Illness checklist (OPCRIT) with a lifetime perspective.

Results: We found a strong association between CLOCK genotype variants and occurrences of initial ($p = 0.0001$), middle ($p = 0.0009$), and late ($p = 0.0008$) insomnia. In MDD the presence of the C allele was linked with an higher occurrence of initial insomnia, while in BD the same allele was associated with insomnia throughout the whole night and reduced the need for sleep ($p = 0.0074$). Other demographic and clinical features were not found to be related with CLOCK polymorphisms.

Conclusions: Our preliminary observations support the hypothesis that CLOCK genotype has an effect on sleep disturbances in MDD. The association of clinical insomnia and CLOCK*C allele in pts affected by MD suggests the presence of a still undefined interaction between CLOCK variants and other variables linked with MD. In fact, since

the C allele was not associated with sleep disturbance in healthy subjects, it is possible to hypothesize a relationship between "eveningness" associated with the C allele and sleep disregulation in MD and BD.

Keywords Major depressive disorder, Bipolar disorder, CLOCK gene polymorphism, Insomnia

HOW DO GENES EXERT THEIR ROLE? PER3 VARIANTS AND POSSIBLE INFLUENCES ON MOOD DISORDER PHENOTYPES

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Objectives: Complex disorders recognize an influence of multiple liability genes, but on the other hand liability genes exert their influence of a wide range of phenotypes and according to the condition of individuals (healthiness, disease). Human PER3 gene (hPER3) is a clock-relevant member of the PER gene family located on the human chromosome 1, and it is expressed in the hippocampus, piriform cortex, and cerebellum. mPER3 RNA levels exhibit robust circadian variation in the suprachiasmatic nucleus (SCN), like mPER1 and mPER2, but unlike the former two, light pulses do not affect mPer3 RNA levels during subjective night. Some genetic variants have been found to be associated in healthy subjects with the preference for and the concentration of activity in the evening (eveningness), as opposed to the morning (morningness), and to Delayed Sleep Phase Syndrome. We decided to investigate the potential association of PER3 variants with various aspects of the symptoms and character in mood disorder pts.

Results: We found an association between *Per3ex18T/C* and age of onset of the disease ($p < 0.005$). According to item 18A and 18B scores of the HAMD-21, related to diurnal variation of the symptoms during antidepressant treatment, we found that *Per3ex18TC**CC subjects showed a worse symptoms in the evening more frequently than did TT homozygous and heterozygous pts, at baseline and at the second week of treatment ($\chi^2 = 10.27$; $p = 0.03$ and $\chi^2 = 10.2$; $p = 0.03$, respectively). Moreover, we found an association between *Per3ex18ins/del* polymorphism and "novelty seeking" as measured by the Temperament and Character Inventory (TCI) ($p < 0.05$), especially for the subscale "extravagance" ($p < 0.05$). Interestingly, healthy people who showed higher "novelty seeking" were previously found to show "eveningness."

Conclusions: Similarly to the HTTLPR one, a single polymorphism may influence a range of aspects. We hypothesize that subjects with PER3 variants are characterized by eveningness and temperamental novelty seeking. If affected by mood disorders, these features have an impact on the earlier onset of illness and greater diurnal variation during illness episodes. These results could shed further light on the involvement of genes controlling circadian rhythms of various aspects of physiological and pathological mechanisms of the brain.

Keywords hPER3 polymorphism, Mood disorders, Novelty seeking, Age of onset of depressive disease, Diurnal variation

ORAL PRESENTATION SESSION F

SIGNIFICANT AND BENEFICAL CHANGES OF THE QUALITY OF LIFE (QOL) WITH AN OPTIMAL CONTROL OF THE VALUES AND CHRONOBIOLOGICAL PROFILE OF BLOOD PRESSURE (BP)

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Objectives: Hypertension is one of the major health problems worldwide. The present study: a prospective, randomized, controlled one, tries to compare the modifications of the QoL (based on pts declarations) after reaching optimal 24 h BP control.

Methods: 72 pts with mild-to-moderate hypertension were submitted to 48 h ABPM, repeated after the 3 wks "run-in" period with: enalapril maleat (Enap, KRKA) 20 mg/day $n = 18$ pts; verapamil (Isoptin RR 240, KNOLL) 240 mg/day $n = 20$ pts; amlodipine (Norvasc, PFIZER) 10 mg/day 34 pts in single morning administration. If the results of the ABPM were considered unsatisfactory; a second ABPM session was repeated after a 4 wk "run-in" period with the new therapeutic schedule: same drugs in same dosage, but in single vesperal administration. All pts reached optimal BP control (BP values and profile). Pts also completed a QoL questionnaire created in our clinic (following the recommendations of SPILKER B. "Guide to clinical trials") and again after 3 months of treatment by the new medication schedule. The QoL questionnaire was completed separately by both pts and relatives/friends. There were no statistically significant differences (χ^2 test; $p < 0.0005$) between the demographic characteristics of the subjects of the 3 therapeutic groups. The kinds of modifications defining the QoL were: (1) general feeling of well-being (self-perception of degree of well-being and health), (2)

different symptoms (the exact description, severity, duration, and frequency and necessity to treat), (3) sexual function, (4) activity performance, (5) sleep quality, (6) cognitive function (memory and capacity to communicate, make decisions, think, take action), (7) satisfaction with life (hopes for the future), (8) social participation (relations with family, relatives, friends, others; relations in the community, data concerning recreational activities), and (9) emotional stability (dominant feelings; thoughts about the future; intensity of emotions; religious or philosophical beliefs; number of contacts/week, quality of social contacts). We employed the self-administered test, supervised by an investigator who confirmed the subject understood and answered each question. The alternative answers were: +- amelioration; 0 unchanged; − worsened.

Results: QoL scores were as follows per treatment, respectively, for the sum of the patient's answers and sum of wife/husband/friend's answers: Enap +109/ + 82; Isoptin RR 240: +107/ + 91; Norvasc +185/ + 161

Conclusions: The new therapeutic schedule allowed optimal control of the BP profile and values; all pts show good compliance to the vesperal administration, and there was statistically significant amelioration of all the QoL parameters. Pts declared a significant ($p < 0.0005$) better amelioration than their relatives/friends. It's possible that a significant placebo effect occurred by the feeling of better surveillance due to application of a sophisticated device (ABPM), which may explain to some extent the very good result of monotherapy.

Keywords Hypertension, Quality of life, Adverse drug effects, Antihypertensive therapy, Nighttime dosing of antihypertensive medication, Chronopharmacology of antihypertensive treatment

HUMAN ENDOGENOUS CIRCADIAN CARDIOVASCULAR RHYTHM

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Objectives: There exists a robust day/night pattern in the incidence of adverse cardiac events with a peak at ~10:00 h. This peak has been attributed to day/night patterns in behaviors affecting cardiovascular function in vulnerable individuals. However, influences on cardiovascular function from the endogenous circadian pacemaker, independent from behaviors, may also affect cardiovascular function.

Methods: To assess the effect of the endogenous circadian pacemaker on baseline cardiovascular function in humans we measured heart rate (HR), cardiac parasympathetic tone (power of high frequency band in ECG inter-beat interval), cardiac sympathetic tone (isovolumetric ventricular contraction time); urinary epinephrine and norepinephrine; systemic blood pressure (SBP); and circulating cortisol throughout a 'constant routine' protocol (38 h of wakefulness with constant posture, temperature, and dim light, as well as identical snacks every 2 h) in 9 healthy young subjects (mean age, 31 yrs; 7 males). Core body temperature (CBT) was measured to assess endogenous circadian phase. Data were assigned a circadian phase (0 degrees = minimum CBT), normalized among subjects (z-score) and subjected to cosinor analysis for detection of significant circadian rhythmicity.

Results: There were significant endogenous circadian rhythms in most variables, with cosinor amplitudes of hormones between 10% to 60% of the mesor, while the other indices exhibited smaller amplitudes (usually 5% to 15% of the mesor). Peaks in indices of cardiac parasympathetic tone occurred around the time of the usual sleep period (although subjects remained awake); and peaks in the cardiac sympathetic index and cortisol around the usual time of awakening. Catecholamines had a steady increase throughout the usual wake period and declined sharply in the biological night, whereas systemic arterial blood pressure was greatest in the evening.

Conclusion: Our data imply that sympathovagal balance has a significant endogenous circadian rhythm independent of behavior, with a peak around the time of usual awakening. This time corresponds to the most vulnerable time of day for adverse cardiovascular events based on epidemiological data. Thus, in addition to behavioral influences on cardiovascular function, the endogenous circadian system may summate with behavioral effects and thereby contribute to the day/night pattern of adverse cardiovascular events in vulnerable individuals.

Keywords Circadian rhythm, Cardiovascular function, Autonomic nervous system, Sympathetic nervous system, Parasympathetic nervous system, constant routine

Supported by NIH K24 HL76446, HL64815, HL76409, MO1 RR02635

SCREENING OF THE PATIENTS WITH ARRHRYTHMIA FROM HEALTHY SUBJECTS BASED ON THE FRACTAL DIMENSION OF HEART RATE VARIABILITY (HRV) BY AN ARTIFICIAL NEURAL NETWORK

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Objectives: HRV has been shown to exhibit fractal behavior. Fractals are self similar shapes with fractional dimension, and we can use them to find order in the biologic systems which seem to have non-predictive and irregular behavior. The exact relationship between fractal behavior of HRV and the condition of the cardiovascular system and its changes with age and diseases have not well studied.

We used an artificial neural network for screening pts with arrhythmia as a pathologic state based on fractal dimension of HRV.

Methods: We used 200 digital rhythmograms which included 100 healthy subjects and 100 pts with arrhythmia. We calculated the fractal dimension of HRV using Nlyser software (version 3.5). We designed an artificial neural network by Neurosollutions software (version 4), and we used age, gender, and fractal dimension of HRV of 150 rhythmograms (75 healthy rhythmograms and 75 pts with arrhythmia) as input data and the condition of being healthy or having arrhythmia as target for training the artificial neural network. We used the data of 20 rhythmograms (10 healthy rhythmograms and 10 pts with arrhythmia) as a validation set. Finally, after training the network, it was tested using the data of 30 rhythmograms.

Results: The designed artificial neural network could screen healthy persons from arrhythmia pts based on the fractal dimension of HRV with 0.2 mean squared errors. The network was 100% successful in identifying healthy persons, and it was 90.14% successful in identifying arrhythmia pts. It had fault for discriminating the arrhythmia pts in 98.6% of the cases.

Conclusions: Fractal dimension, which is used as a factor showing fractal characteristics of HRV, can be used as a diagnostic tool to screen pts with arrhythmia by artificial neural networks. The results depend on the size of our training set for the artificial neural network. If we develop the training set, the network will be more successful in screening the patients.

Keywords Heart rate variability, Fractal dimension, Artificial neural network, Cardiac arrhythmia

EFFECTS OF THE CIRCADIAN TIME OF ANTIHYPERTENSIVE TREATMENT ON THE AMBULATORY BLOOD PRESSURE (BP) PATTERN OF HYPERTENSIVE PATIENTS (PTS) WITH TYPE 2 DIABETES

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Objectives: The prevalence of a non-dipping BP pattern (<10% reduction in BP during nighttime sleep in relation to daytime mean BP) in type 2 diabetes mellitus (DM) is highly variable among different studies. Moreover, non-dipping in hypertensive pts treated with single morning doses of BP-lowering medication is strongly related to the absence of 24 h therapeutic coverage [*J. Hypertens.* 2002;20:1097–1104]. Accordingly, we studied the impact of the time of therapy on the circadian BP pattern in hypertensive patients with DM.

Methods: We studied 585 hypertensive DM pts (328 men), 60.4 ± 11.2 (mean ± SD) yrs of age. 142 pts were untreated at the time of the study; 236 pts took all their antihypertensive medication upon awakening; and 207 took one of the several antihypertensive medications at bedtime. BP was measured for 48 h by ambulatory monitoring, at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night.

Results: 64.1% of the untreated pts were non-dippers. In treated pts, BP was highly reduced during hours of diurnal activity, but not during nocturnal sleep, as compared to untreated pts ($p < 0.001$). Thus, the percentage of non-dippers among treated pts was increased to 69.8%. Most importantly, 25% of the treated pts were risers (nocturnal BP mean above the diurnal mean). As a function of the circadian time of treatment, 73.3% of the pts who took all their BP-lowering medications upon awakening were non-dippers. This percentage was reduced to 64.7% in pts who took one of the several antihypertensive medications at bedtime ($p = 0.041$).

Conclusions: Results of this study confirm the high prevalence of an altered circadian BP pattern in DM. The extremely high prevalence of risers among treated pts with DM indicates the need to establish a proper chronotherapeutic antihypertensive scheme that not only reduces BP but also corrects the altered circadian profile into a dipper BP pattern, which is associated with low cardiovascular risk.

Keywords Diabetes mellitus, Chronotherapy, Non-dipper hypertension, Circadian rhythm

METABOLIC SYNDROME (MS) IS CHARACTERIZED BY AN INCREASED PREVALENCE OF A NON-DIPPER (ND) PATTERN IN AMBULATORY BLOOD PRESSURE (BP)

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Objectives: The individual components of the MS (according to the ATP-III definition, presence of at least 3 of the following factors: high waist perimeter, increased triglycerides, low HDL-cholesterol, high BP, and high fasting glucose) are associated with left ventricular hypertrophy, diastolic dysfunction, and microalbuminuria. Moreover, ND (<10% decline in the nocturnal relative to the diurnal BP mean) has also been associated with increase in cardiovascular events. We investigated the association between MS and impaired nocturnal BP decline.

Methods: We studied 2116 subjects (962 men), 51.8 ± 13.6 yrs of age (mean ± SD). Among them, 261 were normotensive and 1855 were hypertensive (752 untreated at the time of evaluation). BP was measured for 48 h by ambulatory monitoring at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night.

Results: MS was present in 858 of the subjects (40.5%). In untreated subjects, the prevalence of a ND BP profile was significantly higher in pts with MS (44.4 *vs.* 35.8%; $p = 0.007$). These percentages significantly increased in treated subjects, while the higher proportion of ND pattern in the presence of MS was again statistically significant (55.1 *vs.* 48.1%; $p = 0.021$). The day/night BP ratio was significantly lower in MS pts, both among untreated (10.6 *vs.* 11.6 in pts without MS; $p = 0.019$), as well as in treated pts (7.7 *vs.* 9.3; $p < 0.001$).

Conclusions: Results document a significant increase of blunted nocturnal BP decline in MS subjects. These results corroborate the progressive loss of nocturnal decline in BP with increasing fasting glucose and increasing waist circumference previously documented [*Am. J. Hypertens.* 2004;17:43A].

Keywords Metabolic Syndrome, Non-dipper, Hypertension, Non-dipping blood pressure pattern, Circadian rhythm

THE AMBULATORY BLOOD PRESSURE (BP) PATTERN IN RESISTANT HYPERTENSION (RH) IS MARKEDLY AFFECTED BY THE TIME OF ANTIHYPERTENSIVE TREATMENT

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Objectives: Pts with RH present high prevalence of a non-dipper (ND) BP pattern (<10% decline in the nocturnal relative to the diurnal BP mean) and an associated elevated cardiovascular risk. Recent results indicate that ND is partly related to the absence of 24 h therapeutic coverage in pts treated with single morning doses of BP-lowering medications (*J. Hypertens.* 2002;20:1097–1104). Accordingly, we studied the impact of the time of treatment on the circadian BP pattern in pts with RH.

Methods: We studied 712 pts with RH (367 men), 59.2 ± 11.5 yrs of age (mean ± SD). 309 pts took all their medication upon awakening at the time of evaluation, and 403 took one of there several antihypertensive medications at bedtime. BP was measured for 48 h by ambulatory monitoring, at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night.

Results: RH pts who took one of their several medications before bedtime were characterized by a significant reduction in the 24 h mean of systolic and diastolic BP (3.5 and 2.1 mm Hg, respectively; $p < 0.010$) compared with ones took all their antihypertensive medications upon awakening. This BP reduction was much more prominent during the nocturnal resting hours (5.8 and 3.3 mm Hg, $p < 0.001$). Consequently, the day/night BP ratio was significantly increased (by 2.6 and 2.8 for systolic and diastolic BP; $p < 0.001$) in pts who took one of their hypertensive medications at bedtime. A very high 79.0% of pts who took all their medications upon awakening were ND. This prevalence was significantly reduced to 52.6% in pts who took one of their several antihypertensive medications at bedtime ($p < 0.001$).

Conclusions: In RH pts, pharmacological BP therapy should take into account when to treat with respect to the rest-activity cycle of each pt, therapeutic coverage of the medications, and baseline circadian BP profile of each pt.

Keywords Resistant hypertension, Non-dipper hypertension, Chronotherapy of hypertension

INFLUENCE OF CIGARETTE SMOKING (CS) ON AMBULATORY BLOOD PRESSURE (BP) IN UNTREATED HYPERTENSIVE PATIENTS (PTS) WITH ESSENTIAL HYPERTENSION

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Objectives: The relationship between CS and BP is controversial. Some epidemiological studies have found that smokers have lower clinic and ambulatory BP than non-smokers, while others have found a significant increase in daytime BP in smokers (*J. Hypertens.* 1995;13:1209-1215). The objective of this trial was to investigate the relationship between CS and ambulatory BP in untreated hypertensive pts.

Methods: We studied 1250 untreated pts with grade 1 to 2 essential hypertension (531 men), 48.5 ± 13.7 yrs of age (mean ± SD. 720 pts were non-smokers, 212 were currently smokers, and 318 were ex-smokers for at least 1 yr. BP was measured by ambulatory monitoring for 48 h, at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night.

Results: Smokers were characterized by a significant increase in the diurnal mean and a significant decrease in nocturnal mean of systolic BP as compared to non-smokers ($p = 0.027$). The 24 h mean of systolic BP was thus similar between groups (130.3 mm Hg in non-smokers and 131.0 mm Hg in smokers; $p = 0.466$). Diastolic BP and heart rate (HR) were significantly increased throughout the 24 h in smokers ($p < 0.001$). The double (pressure-rate) product (HR × systolic BP) was also significantly elevated in smokers ($p < 0.001$). Ex-smokers presented BP values slightly higher than non-smokers and significantly lower than smokers. Smoking was significantly associated with a marked increase in the day/night BP ratio. The prevalence of extreme-dippers (>20% decline in nocturnal BP) increased from 4% in non-smokers to 10% in smokers ($p < 0.001$).

Conclusions: CS significantly increases the diurnal mean of systolic and diastolic BP, 24 h mean of HR, and of the double-product, a marker of myocardial ischemia. Moreover, the day/night BP ratio is also significantly increased by smoking towards an extreme-dipper pattern, characterized by an increased risk of stroke. Smoking cessation progressively restores BP values to those found in non-smoker hypertensive pts.

Keywords Blood pressure, Heart rate, Rate pressure product, Smoking, Extreme-dipper, Essential hypertension, ABPM

IMPROVED REPRODUCIBILITY OF THE DIPPER CLASSIFICATION IN HYPERTENSION WITH 48H AMBULATORY BLOOD PRESSURE (BP) MONITORING

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Objectives: Non-dipping (<10% decline in the nocturnal relative to the diurnal mean of BP) has been related to an increase in end-organ injury and cardiovascular events. This definition has been frequently criticized due to the inability to reproduce over time the classification of pts into dippers and non-dippers (*Am. J. Hypertens.* 1998;11:403–409). However, earlier results have demonstrated that reproducibility of diurnal and nocturnal mean BP values is more dependent on duration of sampling than on sampling rate (*Hypertension* 2003;42:619–624). Accordingly, we investigated the reproducibility of a dipper pattern in untreated hypertension as a function of duration of BP sampling.

Methods: We studied 621 untreated patients with grade 1 to 2 essential hypertension (296 men), 44.4 ± 13.5 yrs of age (mean ± SD). BP was measured by ambulatory monitoring (ABPM) for 48 h, at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night, on 2 different occasions within 12 wks. Pts remained untreated during the 12 weeks between evaluations. Reproducibility of the dipper classification was obtained using either all data gathered for 48 h as well as the BP data sampled for the first 24 h of each profile.

Results: Using the data sampled for the first 24 h, 102 dippers on the first profile changed to non-dippers on the second, and 86 non-dippers in the first profile changed to dippers on the second, for a total of 30.3% pts changing their classification within 3 months. When reproducibility was evaluated using all data sampled for 48 h, only 12% of the pts changed their original classification. Most important, the range of the difference between profiles in the day/night BP ratio calculated using data sampled for 24 h was 40 (from 18 to −22), 4 times the difference between an extreme-dipper and a non-dipper.

Conclusions: This study corroborates that a reproducible estimation of mean BP values is mainly dependent on the duration of sampling. This prospective trial demonstrates that a single ABPM for 24 h is insufficient for proper diagnosis of hypertension, identification of dipping status, and evaluation of treatment efficacy. Reproducibility of the dipper classification is markedly improved to a high 88% by extending ABPM for 48 h.

Keywords ABPM, Blood pressure sampling requirements, Non-dipper blood pressure status, Dipper blood pressure status

EFFECTS OF THE CIRCADIAN TIME OF ANTIHYPERTENSIVE TREATMENT ON THE AMBULATORY BLOOD PRESSURE (BP) PATTERN OF ELDERLY HYPERTENSIVE PATIENTS (PTS)

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Objectives: Non-dipping in hypertensive (HTN) pts treated with single morning doses is partly related to the absence of 24 h therapeutic coverage (*J. Hypertens.* 2002;20:1097–1104). In the elderly, as compared to young pts, the day/night BP ratio is diminished due to a progressive increase in nocturnal BP with aging. Accordingly, we studied the impact of antihypertensive treatment and the time of therapy on the circadian BP pattern in elderly HTN pts.

Methods: We studied 1286 elderly pts with grade 1 to 2 essential HTN (573 men), 68.4 $\pm$ 5.6 yrs of age (Mean $\pm$ SD). 352 pts were untreated at the time of the study, 547 pts took all their antihypertensive medication upon awakening, and 387 took one of their several antihypertensive medications at bedtime. BP was measured for 48 h by ambulatory monitoring, at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night.

Results: 62.5% of the untreated pts were non-dippers. In treated pts, BP was highly reduced during the hours of diurnal activity, but not during nocturnal sleep, compared to untreated pts. The percentage of non-dippers among treated pts was increased up to 66.3%. As a function of the circadian time of treatment, 75.2% of the pts who took all their medications upon awakening were non-dippers. This percentage was significantly reduced to just 53.7% in pts who took one of their antihypertensive medications at bedtime ($p < 0.001$).

Conclusions: Antihypertensive therapy, mostly given exclusively upon awakening, significantly modifies the circadian pattern of BP. In the elderly, pharmacological therapy should take into account when to treat with respect to the rest-activity cycle of each pt, as a function of the therapeutic coverage of the medications, and the baseline circadian BP profile of the pt.

Keywords Non-dipper blood pressure pattern, Chronotherapy of antihypertensive therapy, Elderly

SYMPOSIUM VI-GENDER ISSUES IN CHRONOMEDICINE

GENDER-BASED APPROACH TO IMPROVE HEALTHCARE QUALITY WORLDWIDE

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In the past 2 decades considerable progress has been made toward improving the quality of women's health and health care. There has been a growing recognition that the biological differences between women and men go beyond reproduction. Genetic, hormonal, and metabolic influences play a role in male and female patterns of morbidity and mortality. A considerable amount of evidence shows that clinically relevant sex/gender and racial/ethnicity differences exist in the pharmacological response to certain drugs. Such differences can impact drug use and safety. Also, a growing body of evidence shows sex differences exist in the incidence, symptoms, and prognosis of numerous diseases/conditions such as HIV/AIDS, tuberculosis, autoimmune diseases, diabetes, and coronary heart disease. Moreover, socially constructed gender differences influence women and men's potential for a long and healthy life. Gender inequalities in income and wealth make women especially vulnerable to poverty and limit women's access to health services. Worldwide, millions of women continue to be deprived of basic health care as a result of poverty and discrimination. Gender bias is particularly observed in the context of medical research, where studies have shown that women have too often been excluded from clinical trials. To eliminate inequalities in care and improve the quality of health services and quality of life for both women and men, the complexity of links between biological sex, social gender, and health must be addressed. Current best practices and/or models of care will benefit from findings revealed by gender-based research. As we learn more about the reasons why inequalities in health care occur, the role of the biological and social factors will be clarified and more research questions will surface. Research findings have the potential to bring about policy changes that will allow the practice of a more focused health care designed to meet the specific needs of both women and men across racial and ethnic groups. The gender-based approach provides a broad range of research opportunities and consequently the chance to improve treatment and disease outcomes for all worldwide: women, men, the poor, the rich, minorities and non-minorities, and most importantly, the children who one day will be the next generation of men and women.

Keywords Gender-based research, Medication safety, Women's health

The views expressed in this abstract are those of the authors and do not necessarily represent the views of the Agency for Healthcare Research and Quality or the U.S. Federal government

GENDER-RELATED DIFFERENCES IN OPTIMAL DOSE AND DELIVERY SCHEDULE OF CANCER CHEMOTHERAPY: SHOULD THE CHRONOTHERAPY SCHEDULE DIFFER BETWEEN MEN AND WOMEN?

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Background: Finding the optimal delivery schedule of anticancer therapy has been the mainstay of developmental therapeutics, in addition to the identification of new agents. Matching the delivery schedule of arabinosylcytosine to the proliferation kinetics of the tumor cured leukemic mice otherwise dying from disease progression under other schedules of delivery. Adjusting this "optimal" schedule to the circadian timing system of mice, so called chronotherapy, further doubled the cure rate of leukemic mice (Haus et al., Science, 1977). Chronotherapy has been at the forefront of the doubling of median survival in pts with metastatic colorectal cancer (MCC) over the past 10 yrs (Lévi, Lancet Oncology, 2001). In 2 multicenter randomized trials, the chronomodulated delivery of 5-fluorouracil, leucovorin, and oxaliplatin infused for 5 days every 3 wks (chronoFLO5), achieved both a better objective tumor response rate ($p < 0.001$) and a better tolerability ($p < 0.001$) than the constant rate infusion of the same drugs. Yet, median survival was 17 months (mo) in both arms (Lévi et al., *J. Natl. Cancer Inst.*, 1994; *Lancet*, 1997). Another "non-chrono" schedule of the 3-drug combination also provided good activity and acceptable tolerability: the FOLFOX-2 regimen consisted of a 2 h infusion of oxaliplatin on day 1, followed by a 44 h infusion of 5-FU, combined with 2 h infusions of LV on days 1 and 2 (De Gramont et al., *Eur. J. Cancer*, 1997). Our group has compared chronoFLO4 to FOLFOX2 with regard to survival in a prospective randomized trial involving 564 pts at one of 36 institutions from 10 countries. No difference in survival was found between both treatment arms (FOLFOX2, 18.7 mo; chronoFLO4, 19.6 mo, p from Log Rank = 0.54) (Giacchetti et al., *Proc. ASCO* 2004). Gender-related differences characterize colorectal cancer incidence, gene expression patterns, as well as 5-fluorouracil (5-FU)

pharmacology (dihydropyrimidine dehydrogenase activity, 5-FU clearance, and circadian rhythm amplitude — lower in females (F) than males (M)). Interestingly, all the preclinical studies of oxaliplatin were performed in male mice.

Methods: The prognostic and predictive value of gender for effect of chronoFLO4 on OS was tested using the log-rank test and Cox proportional hazards regression model. Analyses further assessed gender prognostic and predictive values for Progression Free Survival (PFS), Objective Response (OR), and Overall Toxicity (OTOX: occurrence of any grade 3 to 4 main toxicity per pt).

Results: Gender was not a prognostic factor for OS ($p = 0.11$), PFS, or OR. F pts displayed significantly higher OTOX than M (72.6% *vs.* 55.9%; $p < 0.001$). Strong gender-schedule interactions were demonstrated for both PFS ($p = 0.008$) and OS ($p = 0.001$). In F, the median OS was 19.3 mo on FOLFOX compared to 16.3 mo on chronoFLO4 (Log Rank, $p = 0.027$, 38% larger risk of death). Conversely, the median survival of M pts was improved by 3.1 mo with chronoFLO4 compared to FOLFOX (21.4 *vs.* 18.3 mo; $p = 0.018$, 25% lower risk of death). The gender-schedule interaction was present in each age and performance status subgroup. The predictive value of gender for schedule effect on OS was confirmed following adjustment for pt characteristics. Potential mechanisms for gender-related differences in cancer chronotherapy will be discussed based on experimental and clinical data.

Conclusions: Gender strongly and significantly predicted for the schedule of chemotherapy to optimize OS. While additional studies are required to identify a better chronotherapy regimen in women, the chronomodulated schedule tested here produced a clear survival advantage over FOLFOX in males (2/3 of the MCC patients). Translational research studies aim at the identification of the molecular mechanisms for these gender-related differences in optimal scheduling. Gender-based differences in circadian pharmacology and circadian time structure need to be carefully explored throughout the development of chronotherapeutic schedules.

Keywords Cancer Chronotherapy, Metastatic colorectal cancer, Gender, Sex, 5-fluorouracil, Leucovorin, Oxaliplatin

GENDER DIFFERENCES IN CHRONOPHARMACOLOGY

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Little is known about drug effects in women compared to men. Sex-related differences *i.e.*, male *vs.* female, are generally documented in pharmacokinetics (bioavailability, distribution, metabolism, and elimination) or in pharmacodynamics (gender-related differences in receptor density and sensitivity). The problem of under-representation of women in clinical trials also occurs in chronopharmacological studies where gender differences were not largely investigated. Gender-related differences in biological rhythms involved in chronopharmacology have been documented. The chronopathology of diseases, such as cephalalgia, epilepsy, asthma, etc, well illustrates gender differences. Nevertheless, most of the data concern chronopharmacokinetic differences related to drug bioavailability, distribution, protein binding, hepatic metabolism, and renal elimination. Some chronopharmacodynamic studies have documented gender differences in drug effect in animals as well as human beings. The influence of female-specific issues, such as menstrual cycle, oral contraceptive use, pregnancy, and menopause (influence of endogenous or exogenous administered hormones, oral contraceptives, and post menopausal estrogen) is another aspect which has been more extensively investigated from a chronopharmacological point of view. The clinical significance of such differences remains to be determined.

Keywords Chronopharmacology, Pharmacokinetics, Pharmacodynamics, Gender medicine, Women

THE DEVELOPMENT OF THE HUMAN TIME STRUCTURE FROM CHILDHOOD TO SENESCENCE

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The human time structure consists of rhythms of multiple frequencies, which are superimposed upon each other and upon trends like development and aging. In the human fetus rhythms in numerous body functions are detected which in the circadian range are largely transmitted from the mother presumably via hormonal messages through the placenta. These rhythms determine in animal experiments the fetal susceptibility to toxic insults and the incidence and severity of resulting embryopathies. In the newborn ultradian rhythms predominate in prominence over the circadian rhythms which establish themselves during the first weeks and months of life and reach adult prominence only later in childhood. In healthy adults higher frequency rhythms (*e.g.* pulsatile hormone secretions), ultradian rhythms (a few hours), circadian

(about 24 hs), circaseptan (about 7 days), and circannual rhythms (about a yr) form the complex human time structure which in its interactions between rhythmic variables and with the environment provides for the optimal function of the human organism. With advancing age changes in the human time structure occur some of which may be related to the disturbances seen frequently in aging individuals. Many circadian rhythms which are prominent during adulthood show a reduction in amplitude which may be due to a decrease in peak concentrations of hormones (*e.g.*, melatonin, growth hormone, *etc.*) or an elevation during the circadian trough to compensate for target organ failure (*e.g.*, gonadotropins, TSH). The timing of circadian rhythms (the acrophase) in the elderly becomes more variable and some characteristic changes in timing take place. Some rhythms tend to peak earlier while others show a phase delay. *E.g.*, the maximal urine excretion moves into the night hours contributing to the sleep disturbances experienced by the elderly. The marked decrease in the evening rise in melatonin concentrations removes one messenger providing time information to peripheral tissues. Changes in the usual and probably optimal time relations between rhythms occur (internal desynchronization) and may contribute to dysfunction. With the decrease in circadian rhythm amplitude (*e.g.*, in blood pressure) ultradian rhythms become again prominent leading *e.g.* to the double peak in blood pressure seen in the elderly. The tolerance to phase shift (*e.g.*, shift work) and the capability for phase adaptation after a change in environmental synchronizers (*e.g.*, after transmeridian flight) are impaired. Free running of circadian but also of circannual rhythms has been observed in the elderly. Rhythm disturbances seen in the elderly may lead to malfunction or may be a consequence of chronic disease states occurring in these age groups. Healthy and active elderly subjects maintain their rhythmicity in many frequencies and variables into old age. Attempts to maintain the time structure of the elderly are presently mainly a function of maintenance of an active lifestyle with adequate and time regulated rest periods. Hormonal substitutions (human growth hormone, gonadal hormones, dehydroepiandrosterone, melatonin) given according to their usual circadian rhythm phase have been advocated by some but have not yet had adequate and controlled trials to allow a judgment. Bright light treatment in the morning hours with and without melatonin in the evening prior to the desired sleep time have shown some beneficial effects upon circadian rhythm regulation, well being and in some reports also on cognitive functions in patients with age related rhythm disturbances with and without dementia.

Keywords Human time structure, Growth and development, Infancy, Aging, Internal synchronization

MENSTRUAL CYCLE PATTERNS IN DISEASE AND BEHAVIOR OF MEDICATIONS: METHODOLOGICAL ISSUES

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The occurrence and intensity of many medical conditions exhibit menstrual cycle variation. The signs and symptoms of allergic, arthritic, cardiac, digestive tract, dermatologic, metabolic, neurological, psychological, ocular, and sleep disorders, for example, typically are more intense (or occur only) at the end of the menstrual cycle than around ovulation. Many of these same medical conditions also evidence circadian rhythms. The symptoms of asthma, osteoarthritis arthritis, and certain epilepsies are more likely and more intense during the night than day; while, those of allergic rhinitis, depression, and rheumatoid arthritis are more intense in the morning. Some medical conditions also display marked seasonal differences. Studies show women consume a greater number and diversity of over-the-counter and prescription medications than men. In the past, assessment of the safety and efficacy of medications entailed the conduct of clinical trials primarily involving male subjects; women, especially those in the reproductive life stage, usually were precluded from participating if deemed to be at risk of becoming or being pregnant. Moreover, trials were (and still today continue to be) done without consideration of the biological timing (*e.g.*, morning *vs.* evening) of medications. Thus, the safely and efficacy data of all currently marketed medications are mainly representative of men, one specific administration-time, and one particular season. The safety and efficacy of medications in women of all ages and life stages have yet to be properly explored for their safety and therapeutic value, even now when regulations call for the representative inclusion of women in clinical trials. A growing number of studies demonstrate the pharmacokinetics (PK) and pharmacodynamics (PD: desired and adverse effects) of a wide variety of medications can depend on the circadian time of administration. Only a very few studies have explored the role of menstrual cycle stage on the PK and PD of medications commonly used by young women and still fewer studies have stated information on whether or not subjects were concomitantly taking oral contraceptive agents. From a public health and chronobiological perspective, there is a paucity of critically important information on the safety and efficacy of a variety of medications used by 50% or more of the world's population—women! Future trials must obtain needed safety and efficacy data for women. Women tend to consume a greater diversity and number of medications than men and

many chronic medical conditions experienced by women vary in intensity or manifestation according to circadian, menstrual cycle, and seasonal time. As a minimum, future trials must require more extensive assessment protocols to adequately explore the desired and adverse effects of medications in women when dosed in the morning, at the commencement of the daily activity span, *vs.* evening over one or more menstrual cycles.

Keywords Menstrual cycle, Pharmacokinetics, Pharmacodynamics, Women's health, Chronopharmacology, Chronopathology

PLENARY LECTURE

SLEEP, CIRCADIAN RHYTHMS AND THERMOREGULATION

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All life has evolved in an environment with ~24 h rhythms in light and temperature. This has led to the evolution of circadian rhythms (*circa* = about, *dies* = day) at all levels of functioning, from gene to behavior, including sleep and temperature regulation. Rhythms are internalized and are expressed even when the organism is artificially deprived of all environmental cues about light and temperature. In mammals, including humans, an endogenous biological clock is present in the hypothalamic suprachiasmatic nucleus (SCN). However, this does not mean that, under physiological conditions, cycles of environmental light and body or environmental temperature are irrelevant or redundant. On the contrary, the biological clock is sensitive to changes in environmental light and temperature. We have focused on the sensitivity of sleep-wake rhythm regulating brain areas, including the SCN and preoptic area, to subtle changes in core and skin temperature. Sleep and body temperature are closely related. If animals or humans are exposed to high or low ambient temperatures, the activation of behavioral and autonomic thermoregulatory responses is incompatible with sleep. On the other hand, sleep pressure is closely correlated to the more subtle changes in core and skin temperature that are enforced by the circadian timing system even under constant and thermoneutral ambient conditions (McGinty et al., *Arch. Ital. Biol.* 139:63–75, 2001; Raymann RJEM et al., *Am. J. Physiol.*, in press but on-line accessible). Several studies show sleep pressure is increased during the circadian period of heat loss, when skin temperature is elevated resulting in heat loss, and consequently core and brain

temperature is *decreasing*. Especially the latter seems in contrast with experimental studies demonstrating that *increasing* the temperature of the preoptic area in the brain induces sleep (McGinty et al., *Arch. Ital. Biol.* 139:63–75, 2001). We have proposed that this contradiction may be due to the fact that the *locally* neurons heated in these experiments may under normal conditions be activated by input resulting from an increase in *skin* temperature (Van Someren EJW, *Chronobiol. Int.* 17:313–354, 2000). We tested the hypothesis that sleep may be favored by a subtle increase in skin temperature within the thermoneutral zone, *i.e.*, not activating thermoregulatory mechanisms and mimicking the natural increase in skin temperature that occurs during sleep. Sleep depth and sleep onset latencies were obtained throughout the 24 h cycle while subjects were alternatingly and slowly mildly warmed (35.5°C) or cooled (34.7°C) by means of a thermosuit connected to a computer-controlled water bath. We showed that a mildly warmer proximal skin temperature accelerated sleep onset (McGinty et al., *Arch. Ital. Biol.* 139:63–75, 2001) and increased the probability of deeper sleep stages and decreased the probability of wakefulness. In aged subjects, the decrease in wakefulness was most marked in the early morning. Our findings confirm the idea that age-related changes in thermoregulation throughout the 24 h (Van Someren EJW et al., *Aging Research Reviews* 1:721–778, 2002) are involved in poor sleep, and suggest that sleep may be improved by manipulations of thermoregulatory mechanisms (Van Someren EJW *J. Therm. Biol.* 29:437–444, 2004).

Keywords Sleep, Thermoregulation, Aging, Proximal skin temperature, Body temperature, Human beings, SCN, Circadian rhythm

SYMPOSIUM VII-CHRONOBIOLOGY OF SLEEP AND FATIGUE DISORDERS

MANY ROADS CAN LEAD TO SLEEP-WAKE SCHEDULE DISORDERS (SWSD)

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Introduction: SWSD—delayed sleep phase syndrome (DSPS), advanced sleep phase syndrome (ASPS), and free-running sleep-wake cycle (FR)—have multiple etiologies that can be conceptualized in terms

of the circadian system; is the disturbance in the suprachiasmatic nucleus (SCN) clock itself, in its afferents, or efferents?

Light input: There is a high incidence of SWSD in blind persons and in those with retinopathies or cataracts, where light transduction to the SCN is decreased. Low environmental light conditions (low zeitgeber strength) may contribute to the three-fold higher incidence of "owls" than "larks" in today's western society. In older subjects, low evening illumination contributes to advanced sleep and melatonin phase; low daytime light levels are correlated with low nocturnal melatonin secretion.

Social zeitgebers: Non-photic zeitgebers and social cues are important for regular sleep-wake cycles. Lack of social contacts can indirectly reduce daily light exposure and lead to DSPS. Psychosocial factors may be responsible for transient DSPS in adolescence. Constantly or irregularly changing photic and non-photic zeitgebers have an enormous effect on subjective and objective well-being and health. The "war of the zeitgebers" has its toll in sleep difficulties characteristic of shift-work. Transmeridian flight results in similar symptoms ("jet lag").

SCN clock function: SCN dysfunction can occur at the neurotransmitter or clock gene level. In aging and dementia, the circadian timing system decreases in amplitude. Familial forms of ASPS or DSPS have been found with allelic mutations in one or other of the clock genes. Even in a normal population, clock genes differ between morning and evening chronotypes.

SCN output: The SCN signal may be reduced, or the transduction mechanism (to the relevant physiological systems) impaired. A diminished SCN signal loosens coupling between peripheral clocks (enhancing the probability of internal desynchronization). Non-photic zeitgebers, such as eating habits and activity, modify SCN output and are either in conflict with it or help consolidate stable phase relationships. Thus, although many roads lead to SWSD, a general principle of increasing zeitgeber strength (light, melatonin, and regular daily social and physical activity) can aid entrainment.

Keywords Zeitgeber strength, Light, Melatonin, Sleep-wake disorders, Sleep-wake cycles, Aging

THE RELATIONSHIP BETWEEN SLEEP AND FATIGUE IN BREAST CANCER (BC)

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Background: Fatigue is one of the most common and distressing complaints among cancer pts, not only during radiation and chemotherapy, but also for months to years after completion of treatment. Fatigue interferes with daily life, reduces quality of life, and is often a significant reason why pts discontinue treatment. We hypothesized that some of the fatigue may be related to disrupted sleep, circadian rhythms, and low light exposure. The main objective of this study was to investigate the association between these variables among BC pts.

Methods: As part of a larger, ongoing prospective study on fatigue, sleep, and circadian rhythms in BC pts, we conducted an analysis of 85 women newly diagnosed with stage I-IIIA BC and scheduled to receive 4 cycles of adjuvant or neoadjuvant anthracycline-based chemotherapy. Data were collected before and during weeks 1, 2, and 3 of cycle 1 as well as cycle 4 of treatment. Each pt had sleep-wake activity and light exposure recorded with an Actillume (Ambulatory Monitoring, Inc) for 72 consecutive h and each filled out sleep (Pittsburgh Sleep Quality Index), fatigue (Short Form of Multidimensional Fatigue Symptom Inventory), depression (CES-D), and functional outcome (FACT-B and Functional Outcome of Sleep Questionnaire) questionnaires.

Results: During baseline, on average, the women slept for about 6 h/night and napped for over 1 h during the day. Sleep was reported to be disturbed and fatigue levels were high. Circadian rhythms were robust, but women who were more phase-delayed reported more daily dysfunction ($p < 0.01$). During chemotherapy, circadian rhythms became desynchronized, specifically in measures of amplitude, minimum, slope, and F-statistic. Both fatigue and circadian rhythms improved by week 3 of cycle 1. More fatigue was associated with a more desynchronized rhythm, less activity both during the day and night, and a more delayed phase. During chemotherapy, there were also significant correlations between fatigue levels and light exposure ($r = -0.28$ to -0.45) within both cycle 1 and cycle 4, such that higher levels of fatigue were associated with less light exposure. Changes in light exposure and changes in fatigue within the first 2 weeks of each cycle were significantly correlated ($r = -0.28$ to -0.52).

Conclusions: The data of the current study suggest that the women with BC likely experience both disturbed sleep and fatigue before the beginning of chemotherapy. Although their circadian rhythms are robust, BC pts with more delayed rhythms experience more daily dysfunction secondary to fatigue. Once chemotherapy began, increased fatigue was significantly correlated with decreased light exposure among these pts. Although cause and effect cannot be determined from these data, fatigue and sleep, and fatigue and circadian rhythms were significantly related. Studies evaluating the effect of synchronizing circadian rhythms on fatigue during chemotherapy may be warranted.

Keywords Breast cancer, Fatigue, Sleep-wake circadian rhythm, Circadian phase, Light

Supported by NCI CA85264, M01 RR00827 and the Research Service of the Veterans Affairs San Diego Healthcare System.

MELATONIN FOR CHILDREN WITH DELAYED SLEEP PHASE SYNDROME (DSPS)

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Introduction: Children with DSPS suffer from chronic sleep loss. We present our experience of a long-term follow-up study of children with DSPS, including assessment of cognitive performance and melatonin treatment.

Methods: 45 nondisabled children and adolescents (11 girls and 34 boys, aged 12.6 ± 3.2 yrs) diagnosed as having DSPS were retrospectively reviewed. In 26 of the children, cognitive performance was examined using the Test of Variables of Attention (TOVA).

Results: All had difficulty in falling asleep, and 93% experienced difficulty in waking up. 82% were frequently late for school, 74% were academic underachievers, and 89% had behavioral problems. 79% complained of excessive daytime somnolence. 44% were previously diagnosed as having attention deficit hyperactive disorder (ADHD), and different patterns of association between actigraphic and TOVA variables were observed in those with *vs.* without ADHD. All 45 children received melatonin. Doses ranged from 0.5 to 6 mg (4.5 ± 1.2 mg), and the duration of treatment ranged from 6.1 ± 4.0 months, (max, 16 months). No significant side effects were observed. Falling asleep time and sleep duration significantly improved during melatonin treatment (22:19 ± 01:08 h *vs.* 24:37 ± 01:34 h ($p < 0.001$) and 08:38 ± 01:00 h *vs.* 06:20 ± 01:57 h. respectively; $p < 0.001$). School attendance, academic achievement, and behavior improved substantially in 80% to 89% of the children.

Conclusions: Children with DSPS suffer from socio-behavioral and school problems. Melatonin seems to be efficacious and safe and improves the related problems.

Keywords Delayed sleep phase syndrome, Circadian rhythm sleep disorder, Children, Test of variables of attention, Melatonin

SLEEP-WAKE SCHEDULE DISORDERS [SWSD]: MEDICAL AND OCCUPATIONAL CONSEQUENCES

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In humans, sleep and wake episodes occur at regular times that match the 24 h day-night cycle. The regulation and maintenance of a normal sleep-wake rhythm is achieved through the action of the circadian timing system. When one of its components or the interaction between them is disrupted, sleep-wake patterns can become chronically impaired, leading to a group of primary disorders called Circadian Rhythm Sleep Disorders (CRSD). In persons with CRSD, sleep episodes occur at inappropriate times, often causing wake periods to occur at undesired times. This misalignment between the timing of the individual's sleep-wake rhythm and the 24 h social and physical environment can lead to several unfortunate consequences. These people often fail to adjust to environmentally imposed schedule, such as regular office hours or school timetable, which is likely to result in constant frictions with superiors, difficulty to keep a steady job, dysfunctional behaviors, and academic underachievement. Further, lifetime history of social and functional difficulties consequent to CRSD might pre-dispose persons to develop learning disabilities and personality disorders. Finally, functional consequences of CRSD are frequently misinterpreted as symptoms of psychopathology and accordingly mistreated with psychoactive medications. Several case histories are presented to demonstrate the various medical and occupational consequences of CRSD, which will also highlight the urgent need to raise the awareness of CRSD on the part of health professionals.

Keywords Circadian rhythm sleep disorder

SYMPOSIUM VIII-SHIFT WORK AND HEALTH CONSEQUENCES

SLEEPING BEHIND THE WHEEL: A MULTICAUSAL EVENT

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Issues that attracted our attention when analyzing the truck driver population are the high prevalence of sedentary habits, inadequate diet, and

obesity. All of these are risk factors to some sleep disruptions, which can lead this population to fall asleep behind the wheel. Also, irregular working hours change sleep-wake time arrangements which in turn might affect the ability to drive safely. There are several factors which might cause sleepiness in truck drivers during work. Results of 4 studies on truck drivers are presented. The first one investigated the eating habits of 43 truck drivers; the second compared the effect of working hours on the sleep-wake cycle of 37 truck drivers; the third study evaluated the truck drivers' sleepiness using the Karolinska Sleepiness Scale, and the fourth study evaluated the risk for obstructive sleep apnea among 10,101 truck drivers. The studies revealed that many truck drivers working in an irregular time consume more caffeine and other stimulant substances (such as amphetamines), to mask drivers' sleepiness. The results also suggest an influence of working hours on sleep-wake cycle shown by the alteration of sleep patterns in irregular shift groups. Moreover, the risk of obstructive sleep apnea was high, affecting 26.1% of the truck drivers. These studies confirm the need to study risk factors for sleepiness among truck drivers.

Keywords Truck drivers, Sleepiness, Shift work, Obstructive sleep apnea

Supported by FAPESP (98/13053-3)

SLEEP DISTURBANCES AND SLEEPINESS AMONG BRAZILIAN SHIFTWORKERS

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We evaluated the effects of shift work on sleep in those with quite different duties, such as professional bus drivers and personnel working in oil and gas offshore installations. Concerning the latter and despite some evidence that offshore living conditions are conducive to circadian adaptation (*e.g.*, windowless rooms, and daytime sleep undisturbed by social/domestic demands), offshore shift workers ($n = 93$) reported poor sleep more frequently than daytime workers ($n = 86$), as well as habitual difficulty in falling asleep, fragmented sleep, short sleep episodes and feeling tired upon awakening. Sleep loss due to circadian rhythm disruption is not the only factor that induces sleepiness in shift workers and sleep disorders also play an important role. Our polysomnographic findings on bus drivers ($n = 32$) show that a considerable proportion of them (38%) met

the criteria for apnea-hypopnea syndrome and periodic leg movements (28% during diurnal sleep). Multiple Sleep Latency Tests conducted on the bus drivers during the day revealed 42% met the criteria for excessive sleepiness and the same texts done during the night found 38% met such criteria. An association between circadian disruption and sleep disorders will clearly act synergistically, with deleterious consequences for public and individual health and safety. Consequently, it is important that shift workers and schedulers be aware of the importance of adequate sleep, and of the dangers of inverted working and rest periods.

Keywords Sleepiness, Shift workers, Multiple sleep latency tests, Bus drivers

Supported by AFIP and FAPESP/CEPID (98/14303-3)

LONG WORKING TIMES: IS THIS A PROBLEM? STUDIES ON PETROCHEMICAL AND HEALTHCARE SHIFTWORKERS

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Introduction: Implementation of 12 h shifts is a controversial decision for managers and scientists. A worldwide trend in chemical and petrochemical industries is to extend the duration of shifts. In Brazil, a traditional work schedule still adopted by hospitals is fixed 12 h day or night shifts followed by 36 h off, and several Brazilian petrochemical companies want to implement or are already working 12 h shifts.

Objectives: To compare the duration and quality of sleep and alertness in petrochemical and healthcare shift workers.

Methods: 22 petrochemical workers, all males, and 49 healthcare workers of a university hospital, mostly females, participated. Data was collected in 1999 and 2000. For 2 consecutive wks workers filled out sleep logs and 10 cm visual analog scales to evaluate sleep quality and alertness. Perception of alertness was self-evaluated 3 to 4 times during the 12 h day and/or night shifts.

Results and Discussion: Although the workers were males and females performing very different jobs in distinct work settings, results were similar in the 2 study groups. The duration of sleep was reduced and worse during when taken during the daytime hours compared to when taken during the nighttime, and perception of alertness decreased as the work progresses during the nighttime.

Conclusions: These studies confirm the importance of the features of the shift work schedule and the work organization for workers' health, well-being, and safety.

Keywords Shift work, Sleep, Alertness, Petrochemical workers, Healthcare workers

PROMOTING PERFORMANCE IN SHIFTWORK BY LIGHT, SLEEP, AND NUTRITION

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Objectives: To improve performance a series of the countermeasures were evaluated such as a changed sleep regimen including naps, light treatment, and meal content and meal timing.

Results: The shift work studies performed within our research group and others have clearly shown that the major obstacle for achieving a good recuperation is a short rest periods (<10 h) between shifts and forced early morning awakenings (before 06.00 h). The introduction of rest periods, including naps at work during prolonged awakenings in transport (for example long-haul flights), military, and public healthcare, have been shown to improve performance The introduction of light treatment in shift work is rapidly increasing in order to elevate alertness at work and to promote a night work adaptation or re-adaptation to a daytime orientation of the circadian rhythm. We recently demonstrated that bright light exposure during rests on the night shift in industrial settings is sufficient to reduce sleepiness and melatonin at night. But it is evident that bright light exposure is less efficient in other light/dark circumstances, such as on oil platforms. The effect of nutrition on performance seems to be weaker compared to the circadian influence. But the effect is significant depending on macronutrient composition or drastic withdrawal of food (fasting). The timing of food intake has less acute influence on performance according to our own data.

Conclusion: Appropriate use of countermeasures—including light, sleep, and nutrition—can significantly improve performance in shift work.

Keywords Bright light, Meal, Nap, Night work, Performance, Shift work

ORAL PRESENTATION SESSION G

CIRCADIAN RHYTHMS IN SUBJECTIVE VARIABLES OF SHIFT WORKERS FOLLOWING SHUTDOWNS

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Objectives: Attempts were made to study the effects of frequent shutdowns of a cement factory on circadian rhythms in subjective drowsiness (SDr), fatigue (SF), and attention (SA) of shift workers.

Methods: In 40 subjects that included 20 shift workers, SDr, SF, and SA were measured at least 4 to 6 times per day for over a period of 1 wk. The rotation of shifts was from night (00:00 to 08:00 h) to evening (16:00 to 00:00 h) and to morning shift (08:00 to 16:00 h), for 8 h/day for 1 week per shift. At the time of this study the shift workers had already experienced about 30 months (mo) of slowing down of industrial activity that included 8 mo of near complete shutdowns. Nonetheless, during this period shift workers were assigned shift duties, irrespective of workload and activity of the factory. However, whenever workers were on night shift, they slept (about 7 h/day for 6 days) in their workplace. Data were analyzed with the help of cosinor rhythmometry and power spectrum analysis.

Results: Statistically significant circadian rhythms were validated for all subjective variables in most of the shift workers. The findings were comparable with those obtained for the day workers. Circadian periodicity ($\tau = 24$ h) appears to be the dominant feature for all three variables in day and shift workers. In addition, the phenomenon of frequency multiplication was also witnessed in SDr and SF. Few subjects belonging to both categories exhibited 12 h and 8 h periods in the rhythm of their subjective drowsiness and fatigue. However, in these cases the next prominent period was found to be that of 24 h. Further, all subjects (both day and shift workers) exhibited a sleep-wake rhythm with a τ very close to 24 h.

Conclusions: The observed synchronized rhythms in subjective variables of shift workers could be attributed to the availability of 'on job' sleep, especially because during frequent shutdowns of the factory they slept at their work places during the assigned night shifts and behaved like day workers. The findings of this study seem to have an important bearing on the formulation of strategies to optimize human shift work.

Keywords Shift work, Circadian rhythm, Subjective drowsiness, Fatigue, Industrial slough

"Supported by UGC, New Delhi under DRS-SAP; and DST, New Delhi under Fast-Track and FIST Schemes"

CIRCADIAN RHYTHMS OF THE SPECTRAL CHARACTERISTICS OF UTERINE ELECTROMYOGRAPHY (EMG): A CASE STUDY

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Objectives: Tocodynamometric or electromyographic recordings of uterine contractions (CT) have demonstrated significant circadian rhythms of contraction rate. However, no studies of the uterine electromyogram (EMG) have yet analyzed the 24 h rhythm of the contraction spectral content. The EMG mainly contains two frequency components, the FWL and FWH that could be related to the propagation and excitability of the uterus. This study was performed to explore the changes in the CT spectral characteristics over a 24 h period.

Methods: We recorded continuously, by telemetry, uterine CT on one cynomolgus monkey, from day 160 of gestation until labor. We obtained a continuous 8-day recording of EMG signals. The animal was fed *ad-libitum* and subjected to a 14 h L:10 h D photoperiod. On each EMG signal, all CT were isolated and analyzed. The time-frequency representation of CT was calculated to extract FWL and FWH. Each frequency component was characterized in both energy and frequency terms. Data were compared with Kruskal-Wallis and Wilcoxon Mann-Whitney tests, with the 5% level of statistical significance.

Results: We could identify 1043 CT. The analysis of the mean number of CT/h showed a pattern similar to those previously described in the literature (maximum in the dark and minimum in the light period). We also found a circadian variation of the length and of the total energy of CT, but with an opposite phase. For the spectral characteristics of FWL and FWH, we identified different patterns: there was no frequency modulation in FWL, but conversely there was a frequency modulation in FWH (lower frequencies during light than during darkness).

Conclusions: We identified several temporal and spectral circadian variations of CT spectral content. Moreover, the observed significant variations were not the same for the different parameters. However, we shall have to confirm these results on other monkeys and also test the synchronicity of the observed rhythms with the LD photoperiod. These findings suggest that uterine contraction must be recorded for the entire 24 h to

obtain global information on the activity of the uterus, especially for preterm delivery monitoring. Moreover, the circadian evolution of the CT characteristics could suggest that tocolytic treatment efficacy may vary with the time of administration.

Keywords Uterine contractions, Pregnancy, Spectral analysis, Protein biochip, Tocolysis, Monkey

EVALUATION OF CIRCADIAN PHASE-SHIFTING EFFECTS OF RAMELTEON IN HEALTHY SUBJECTS

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Objective: To evaluate the circadian phase-shifting ability of ramelteon, a selective MT_1/MT_2 receptor agonist being studied as a chronosomnotic agent for insomnia treatment.

Methods: In this randomized, 4 period crossover study, 18 healthy subjects received placebo, ramelteon 4 mg, ramelteon 16 mg, and melatonin 5 mg (used as a positive control). Each treatment period consisted of placebo on Night 1, double-blind treatment on Night 2, placebo on Night 3, with a 4 to 11 day washout between periods. Study drug was given at 17:00 h, followed by a 30 min nap starting at 18:00 h monitored by polysomnography (PSG), after which saliva sampling occurred every 30 min (10 samples). Overnight PSG began at 23:00 h. The primary measure was change in dim-light (<20 Lx) salivary melatonin onset (DLSMO) from Night 1 to 3; onset was defined as the time melatonin levels increased to ≥4.0 pg/mL. A phase advance was indicated by the shift of DLSMO to an earlier time.

Results: Compared with placebo, ramelteon produced statistically significant phase advances in DLSMO from Night 1 to 3 (mean difference from placebo: 4 mg, 39.4 min, $p = 0.009$; 16 mg, 32.2 min, $p = 0.031$). Exogenous melatonin also advanced DLSMO to an earlier time ($p = 0.002$), validating the sensitivity of this model. No significant differences in latency to REM or REM-sleep distribution were seen in any treatment group *vs.* placebo. Compared with placebo, ramelteon promoted sleep during Night 2 nap, with statistically significant increases in sleep efficiency (4 mg: 60.5 *vs.* 74.8%, $p = 0.037$; 16 mg: 60.5 vs. 80.4%, $p = 0.004$) and reductions in sleep latency (16 mg: 13.5 *vs.* 7.3 min, $p = 0.025$).

Melatonin did not show sleep-promoting effects for these variables. Adverse event rates were similar for all treatment groups.

Conclusion: Ramelteon produced a phase advance of the endogenous circadian rhythm, as indicated by statistically significant advances in the timing of DLSMO.

Keywords Ramelteon, Phase advance, Phase shift, Dim-light salivary melatonin onset (DLSMO), Insomnia

EFFECTS OF MELATONIN ON THE RESPONSES TO INTERMITTENT EXERCISE

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Objectives: We examined the effects of a single 2.5 mg dose of melatonin on the thermoregulatory and circulatory responses to intermittent exercise at a mean $\pm$ SD room temperature of 27.2 ± 0.4°C, a mean $\pm$ SD relative humidity of 55 ± 3%, and a light intensity of 200 to 300 Lx.

Methods: In a double-blind crossover study, 6 male participants ingested either melatonin or placebo at 11:45 h. Participants rested in a semi-supine position for 75 min and then completed an intermittent exercise protocol involving 66 min of running on a treadmill at alternating intensities of 40, 60, and 80% of maximal oxygen uptake. Repeated measurements of rectal and weighted mean skin temperature (Ramanathan, *J. Appl. Physiol.*, 19:531–533, 1964,), heart rate, blood pressure, skin blood flow, subjective alertness and sleepiness, ratings of perceived exertion (RPE), and thermal strain were recorded as dependent variables.

Results: No effects of melatonin were found on the dependent variables measured during the resting period ($p > 0.10$). After the first 25 min of the subsequent exercise period was completed, melatonin was found to moderate the increase in rectal temperature by approximately 0.25°C ($p = 0.050$) and magnify the increase in skin blood flow by approximately 60% ($p = 0.047$). Mean $\pm$ SD post-exercise systolic blood pressure was 7.8 ± 2.5 mm Hg lower than before the exercise in the melatonin trial; a change which differed significantly to that in the placebo trial ($p = 0.018$). Melatonin did not influence subjective alertness and sleepiness before and after exercise and did not change the responses

of mean skin temperature, RPE, and thermal strain during the exercise ($p > 0.10$).

Conclusions: In summary, it is apparent that a 2.5 mg dose of melatonin has hypothermic, but not soporific, effects during 66 min of intermittent exercise performed in moderate heat stress. Whether such effects improve endurance athletic performance in hot conditions remains to be confirmed. Our data also suggest that post-exercise systolic hypotension is more marked after ingestion of melatonin.

Keywords Thermoregulation, Pineal function, Physical activity, Blood pressure, Blood flow, Melatonin, Exercise

MONTH OF BIRTH AND REPRODUCTIVE PERFORMANCE IN CONTEMPORARY WOMEN

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Objectives: A statistically significant association between month of birth and number of live-born children has been found among 2 839 reproducing women >45 yrs of age, born in Austria in 1946–1956. In contrast, an association with childlessness ($n = 388$) was absent (Huber et al., 2004). In order to reveal a causal mechanism and to determine an association of fecundity and infertility with months of conception, we reanalyzed the original data according to the ovopathy concept.

Methods: Optimal reproductive performance and childlessness are assessed according to the predictions of the seasonal optimal ripening oocyte (SOptRO) and seasonal pre-ovulatory overripeness ovopathy (SPrOO) hypotheses, respectively. The *'prime' and 'minor' SOptRO months* and the *'high risk', 'no risk'* and *'very high risk' SPrOO months* were determined on the basis of *a priori* knowledge of ovulatory and anovulatory seasons in mammals and the general human birth distribution in Europe which coincides with that in Austria in 1946–1956.

Results: The observed numbers of mothers with 2+ children ($n = 899$) born in different months throughout the year were in line with the 'SOptRO' predictions ($\chi^2 = 2.81$; $p = 0.045$). We found an excess of births of childless women during the *'high risk'* SPrOO months and a deficit

during the *'very high risk'* months, interpreted as due to an inherent dose-response inversion. The linear by linear trend of observed numbers in the *'high risk', 'no risk', and 'very high risk'* months was in line with the SPrOO predictions ($\chi^2 = 8.43$; $p = 0.009$).

Conclusion: Fecundability and childlessness are apparently dependent on the gradient of oocyte maturation before fertilization which modulates future constitution and lifetime reproductive fitness.

Keywords Ovopathy, Seasonality, Infertility, Fecundity, Dose-response inversion

CIRCADIAN RHYTHM IN ENDOTHELIAL FUNCTION IN WOMEN UNDER CONSTANT ROUTINE CONDITIONS

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Objectives: The present study was undertaken to investigate the existence of an endogenously generated circadian rhythm in endothelial function, melatonin, blood pressure (BP), and heart rate (HR) in pre- and postmenopausal women and whether these rhythms are altered after menopause.

Methods: Healthy, non-smoking pre-menopausal ($n = 11$) women not on oral contraceptives and postmenopausal ($n = 10$) women not on hormone replacement therapy were studied during a 22 h constant routine (dim light <10 Lx, controlled temperature, posture, meal intake, and maintained wakefulness). Flow-mediated dilatation (%FMD) and glyceryl-trinitrate (GTN)-mediated dilatation was assessed by high-resolution ultrasound of the brachial artery. Salivary melatonin, BP, and HR were measured hourly. Pre-menopausal women were all studied during the follicular phase of the menstrual cycle.

Results: %FMD and%GTN was significantly higher in pre-menopausal women (9.9 ± 1.0%FMD (mean $\pm$ SEM); 18.2 ± 1.8% GTN; $p < 0.01$) compared with postmenopausal women (6.5 ± 0.5% FMD; 11.5 ± 1.6% GTN). A significant day-night variation in % FMD was observed in

pre-menopausal women (day 9.1 ± 0.8; night 10.2 ± 1; $p < 0.05$). This rhythm was attenuated in postmenopausal women (day 6.8 ± 0.6; night 6.0 ± 0.4). No variation over time was observed in GTN-mediated dilatation in either study group. Postmenopausal women had earlier melatonin onsets (melon50% 20.9 ± 0.4 h) and a significantly earlier melatonin acrophase (1.1 ± 0.5 h; $p < 0.05$) compared to pre-menopausal women (melon50% 22.3 ± 0.5 h; acrophase 2.3 ± 0.3 h). Postmenopausal women also had a reduced melatonin amplitude (15 ± 2 pg/mL) compared to pre-menopausal women (21 ± 5 pg/mL), although this decline was not statistically significant. A circadian rhythm in both systolic and diastolic BP and HR was also observed in both pre-menopausal and postmenopausal women. Systolic BP was significantly increased ($p < 0.05$) in postmenopausal women.

Conclusions: The findings show evidence of an endogenous circadian rhythm in endothelium-dependent function (%FMD) in pre-menopausal women. The rhythms in vascular function and melatonin are altered in women after the menopause.

Keywords Endothelial function, Flow-mediated dilatation, Glyceryltrinitrate-mediated dilatation, Blood pressure, Heart rate, Melatonin, Menopause, Women

PREDICTORS OF MENSTRUAL CYCLE LENGTH IN TURKISH WOMEN

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Background: Several studies found correlations between menstrual cycle length and health outcomes, including type 2 diabetes, breast cancer, and hip fractures. It is known that as women approach menopause cycles get shorter. In this study we aimed to investigate some correlates of the recalled menstrual cycle length at ages 20 to 30 yrs in a group of Turkish women from Isparta who were interviewed at ages 45 to 60 yrs for a study on menopause.

Methods: Demographic, anthropometric, and reproductive characteristics were assessed in a questionnaire. Multiple regression analysis was performed to determine predictors of menstrual cycle length. A stepwise model selection algorithm was applied with a significance level of 0.15 for variable entry into the model.

Results: In univariate analysis, longer duration of menstrual flow ($p = 0.0098$), presence of dysmenorrhea ($p = 0.0484$), and lower body weight at 25 yrs of age ($p = 0.0062$) was associated with shorter cycles. In multivariate analysis, menarche age ($p = 0.0314$) and yr of birth ($p = 0.056$) appeared as additional covariates, while dysmenorrhea did not remain in the final model. A later age at menarche was associated with longer cycles; whereas, a later yr of birth was associated with shorter cycles.

Limitations: All menstrual characteristics were self-reported and retrospectively assessed.

Conclusions: Most of current findings of this study are consistent with previous research. To our knowledge, a possible association between yr of birth and cycle length has not been previously reported. Possible secular trends in cycle length deserve further research.

Keywords Menstrual cycle, Year of birth, Body weight, Dysmemorrhea, Menarche age, Menstrual flow duration, Women

CHRONOPHARMACOLOGY OF THE NEUROMUSCULAR BLOCKER ROCURONIUM

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Objectives: Anecdotally, anesthesiologists comment that individual pts show markedly different responses to neuromuscular blockade (NMB) despite accounting for body weight. Clinically, inaccurate prediction of the length of NMB may result in undesirable complications, including insufficient or prolonged paralysis and aspiration. Animal data display a profound circadian variation in the length of NMB elicited by pancuronium. Unlike pancuronium, rocuronium can be used clinically as the single neuromuscular blocker for procedures occurring at all times of the day and night. We aimed to determine the extent to which the time of day of administration of the non-depolarizing neuromuscular blocker, rocuronium, affects the duration of blockade in pts undergoing general anesthesia.

Methods: 40 pts received an initial fat-free-mass related dose of rocuronium (0.6 mg/kg), followed by a second dose (20% of the initial), and a third standard dose (10 mg) during the anesthetic. Neuromuscular function was monitored throughout.

Results: There is a statistically significant difference ($p < 0.05$) in duration of rocuronium NMB between anesthetics conducted in the morning (mean 49 ± 16.18 min) and the afternoon (mean 36 ± 12.6 min). This pattern is consistent with the data previously published on pancuronium in animals. The longest duration of NMB occurs at ~09:00 h and shortest at ~15:00 h.

Conclusions: These data strongly suggest a circadian effect in the duration of NMB elicited by rocuronium in humans. The potential exists to develop an algorithm for rocuronium dosing taking into account body mass and time of the day to enable more accurate prediction of the duration of NMB.

Keywords Rocuronium, Circadian rhythm, Neuromuscular blockade, General anesthesia, Chronopharmacology

GENOMICS AND PROTEOMICS: APPLICATIONS TO CHRONOMEDICINE

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Objectives: In the past few years there has been growing recognition of the importance of circadian rhythms in peripheral organs, such as the heart. Chronocardiovascular paradigms include heart rate, blood pressure, and pathophysiologic processes, such as the early morning timing of myocardial infarctions. Genomics and proteomics are highly sensitive new techniques that have important applications to study these rhythms in health and disease.

Methods and Results: We previously used genomics (Affymetrix microarrays, COSOPT, ANOVA) to examine rhythmic gene expression in normal murine heart, and demonstrated that 1634 genes (~13% of genes analyzed) exhibit 24 h cycling. We now examine gene expression in heart disease, in a model of pressure-overload cardiac hypertrophy induced by aortic banding in mice. We found global patterns of the cycling of gene expression remarkably similar in normal *vs.* disease, with a biphasic distribution that peaks once in the day and once at night. Importantly, we also found rhythmic up-regulation of genes critical to cardiovascular growth, renewal, and remodeling, including matrix molecules (TIMPs, MMPs, integrins, collagens), and clinically relevant biomarkers (ANF, BNP). Additional studies applied proteomics involving surface enhanced laser desorption/ionization time-of-flight mass spectrometry

(SELDI-TOF-MS) for global protein profiling (2 to 200 kDa) and gel electrophoresis and MS to identify specific proteins, from normal *vs.* circadian desynchronized animal models.

Conclusions: In summary, the molecular analysis of circadian rhythms using genomic and proteomic tools can provide important insights into the mechanisms of homeostasis and the pathophysiology of disease. In the next few years, these tools will answer important questions in chronomedicine, and open new avenues for molecular diagnostics, chronotherapeutics, and personalized medicine.

Keywords Genomics, Proteomics, Circadian rhythm, Cardiovascular physiology, Chronomedicine

ROLE OF THE ENDOGENOUS CIRCADIAN SYSTEM IN BRONCHOCONSTRICTION, AIRWAY INFLAMMATION AND RESCUE MEDICATION USE IN ASTHMA

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Objectives: In many pts with asthma, pulmonary function worsens and asthma symptoms increase at night, producing 'nocturnal asthma'. Our aim was to examine the role of the endogenous circadian system—independent of the behavioral sleep/wake cycle—on indices of bronchoconstriction, airway inflammation, and rescue medication use in asthma.

Methods: We studied 15 adult asthmatics throughout 2 complementary protocols while subjects lived in a 'time isolation' laboratory in dim light (<8 Lx): (1) an 8 day forced desynchrony (FD) with recurring 'day' length of 28 h such that all behaviors occurred evenly across the circadian cycle; (2) a 40 h 'constant routine' (CR) with continued wakefulness, constant posture, and identical snacks every 2 h. Indices of bronchoconstriction ([FEV_1] and airways resistance [R]) and airway inflammation (exhaled nitric oxide [NO]) were measured every 2 to 4 h throughout both protocols. Also, the time of each symptom-based bronchodilator rescue medication use (beta$_2$-adrenergic agonist inhaler) was recorded. Core body temperature was used to assess circadian phase and circadian rhythmicity was tested using cosinor analysis.

Results: Results were virtually identical in both the FD and CR protocols. Bronchoconstriction (FEV_1 and R) had significant circadian rhythms

with the worst values during the biological night. The largest circadian peak to trough change occurred for R [mean, 44%]. Similarly, 81% of rescue medication uses occurred across the biological night (50% expected by chance). In contrast, airway inflammation (exhaled NO) had a significant circadian rhythm [mean, 33%] with a peak during the biological day.

Conclusion: Using 2 complementary circadian protocols, we have uncovered a robust influence of the endogenous circadian system (independent of the behavioral sleep/wake cycle) on bronchoconstriction, airway inflammation, and rescue medication use in asthma. The circadian effect on bronchoconstriction rather than airway inflammation appears to be involved in 'nocturnal asthma'.

Keywords Asthma, Circadian rhythm, Sleep, Inflammation, Bronchoconstriction, Constant routine, Forced desynchrony

Supported by NIH grants HL76446, HL64815, HL76409, RR02635

ORAL PRESENTATION SESSION H

SLEEP DISTURBANCE AFTER SCN-PINEAL PATHWAY DISRUPTION IN HUMANS

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Objectives: Melatonin is usually secreted only at night and may influence sleep. We previously found that complete cervical spinal cord injury (SCI) interrupts the neural pathway required for melatonin secretion. Thus, we investigated whether the absence of nighttime melatonin in cervical SCI leads to sleep disturbances.

Methods: We assessed the sleep patterns of 3 subjects with cervical SCI (C4A, C6A, and C6/7A) and without nocturnal melatonin production and 2 control patients with thoracic SCI (T4A, T5A) and normal melatonin rhythms Subjects had a fixed sleep schedule for 1 week followed by an

8 h sleep opportunity in the polysomnography laboratory at the same time of day. We also compared the results of the 2 SCI groups to results from 10 healthy control subjects undergoing a similar protocol.

Results: Subjects with cervical SCI had significantly longer wakefulness after sleep onset (81 [66 to 81] min) as compared to both thoracic SCI (33 [28 to 39] min) and healthy controls (28 [19 to 38] min; $p < 0.05$). There was no difference in the proportion of the different sleep stages, although there was a significantly increased REM-onset latency in subjects with cervical SCI (220 [162 to 277] min) compared to subjects with thoracic SCI (34 [25 to 43] min; $p < 0.05$). The compromised sleep was not systematically associated with sleep apnea or medication use.

Conclusions: Absence of nighttime melatonin in cervical SCI may help explain sleep disturbances in this population. This result suggests a role of endogenous melatonin in normal sleep maintenance in humans. Furthermore, this finding raises the possibility that melatonin replacement therapy could help normalize sleep quality in pts with cervical SCI.

Keywords Melatonin, Polysomnography, Quadriplegia, Sleep, Spinal cord injuries

ACTIGRAPHY OF SLEEP BEHAVIOR: INTERACTIONS BETWEEN GENDER, SEASON AND CHRONOTYPE

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Objectives: The aim of this study was to investigate whether gender, season, or chronotype influences sleep behavior of university students.

Methods: We collected detailed data on the activity-rest pattern of 34 medical students (19 female and 15 male), ranging in age from 19 to 31 yrs, using actigraphy combined with diaries. Subjects were monitored with Actiwatch® Actometers (CNT, UK) for 16 days in May 2003 and again in November 2003. All subjects completed a Horne and Östberg Questionnaire to determine their chronotype. Actiwatch® sleep analysis Software (CNT, UK) was used to assess sleep parameters. Statistical analysis of study data was done by ANOVA.

Results: We found no significant gender specific difference in sleep efficiency, sleep onset latency, and actual sleep time. Furthermore, we found no significant difference in these sleep parameters between week nights and weekend nights. As expected mid-sleep was significantly earlier in morning types (04:08 h, SD = 0:48 h) than in evening types

(04:28 h, SD = 0:48 h; $p < 0.02$) and later during weekends than week days. A difference between morning and evening types was found in sleep efficiency, which was better for morning types (87.9%, SD = 1.30) than evening types (84.3%, SD = 87; $p < 0.008$). As for the seasonal influence, the actual sleep time in winter was found to be significantly longer (mean 6.9 h, SD = 0.13) than in summer (6.6 h, SD = 0.10; $p < 0.02$).

Conclusions: We found chronotype and season influences on sleep behavior. In contrast to Kohsaka et al., (Experientia 48: 316–9; 1991), our subjects had a longer actual sleep time in winter; this, however, is consistent with the findings of other studies like Wirz-Justice et al., (Naturwissenschaften 71: 316–19; 1984), which found shortening of the fraction of sleep in spring compared to the other seasons under free-running conditions. However, we could not reproduce their finding of a gender effect on sleep time. In contrast to the studies cited above, our subjects could carry on with their daily routine during the testing period.

Keywords Actigraphy, Sleep patterns, Chronotype, Season, Gender

THE ENDOCRINE RESPONSES OF SHORT DAY ACCLIMATED SOCIAL VOLES *MICROTUS SOCIALIS* TO LIGHT INTERFERENCE (LI)

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Objectives and Methods: Photoperiod is a major proximate factor mediating seasonal acclimatization of the social voles *Microtus socialis*. We examine the endocrine responses of short day (SD; 8L:16D; lights on between 08:00 to 16:00 h) acclimated voles to LI (SD + LI, 15 min every 4 h, during the dark period. The first LI was given at 19:45 h) for at least three weeks. Both serum corticosteroids and urinary catecholamine levels were determined. Urine was collected over 48 h at 4 h intervals, while serum was collected 2 h before dark. Students' *t*-test was used for establishing significant differences between groups.

Results: Mean serum corticosteroid concentration of SD + LI-voles was higher ($p < 0.001$) than that of SD-voles (33.36 ± 2.32 and 10.14 ± 3.62 ng/mL, respectively). Equivalently, urinary catecholamine content rhythms exhibited elevated values throughout the day and night in comparison to the SD-voles. Mean total urinary catecholamine concentration was increased three-fold in SD + LI-voles compared to SD-voles.

Mean urine volume of SD + LI-voles was 61% lower than that of SD-voles ($p < 0.05$).

Conclusions: The acute response of both the sympathoadrenomedullary and the hypothalamic-pituitary-adrenocortical axis of*Microtus socialis* to LI, implies that LI may act as a stress factor which imposes a great threat to homeostasis with negative effects on the survival of voles in the cold season. Taken together, the data are consistent with the hypothesis that photoperiodic mechanisms that enhance energy allocation for thermoregulation in winter-acclimated voles are disturbed by LI. Thus, these effects partly account for the death of such voles in natural conditions. As a final point, these data are in agreement with recent suggestion of using photoperiod manipulation during ambient darkness as novel chronobiological method for controlling population pest outbreaks in agroecosystems.

Keywords Catecholamine, Corticosteroids, Pest-control, Seasonality, Photoperiod, Light disturbance, *Microtus socialis*, Voles

ASSOCIATION BETWEEN REST-ACTIVITY PARAMETERS AND SUBJECTIVE VARIABLES OF WELL-BEING IN DEMENTIA PATIENTS (PTS)

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Objective: The aim of the present research was to investigate the association between different non-parametric rest-activity parameters and disturbances in cognition, disease progress, competence to perform daily activities, mood, and social interactions in dementia pts.

Methods: 87 women aged 85.5 ± 5.9 yrs (mean ± SD) were studied while living in assisted facilities at 12 different homes for the elderly. Subjects wore an actigraph for 2 wks, strapped on their wrist. To analyze the actimetry profile, the following non-parametric calculations were applied: dichotomy indices, inter-daily stability, and intra-daily variability. Caregiver questionnaires and neuropsychological assessments were applied to obtain the cognitive, functional, behavioral, and emotional state of the subjects, all thought to reflect some aspects of the overall pt well-being. The Spearman's rank correlation test was applied to study associations between rest-activity parameters and subjective variables. Significance was set at $p \leq 0.05$.

Results: A stable rest-activity rhythm, measured with the inter-daily stability parameter, was positively associated with better cognitive functioning. A marked and stable rest-activity rhythm, measured with the dichotomy indices and the inter-daily stability parameters, respectively, were associated with a lower stage of disease progression as well as with less difficulties in performing daily activities. A stable and marked rest-activity rhythm was equally correlated with a better mood and better social interactions/functioning. The fragmentation of the rest-activity rhythm, measured as the intra-daily variability parameter, was not statistically significantly associated with any pt cognitive, functional, behavioral, and emotional scales used in the present study.

Conclusions: Our results indicate that the rest-activity rhythm reflects well-being in dementia. Importantly, our finding that the *stability* of the circadian profile over days showed the strongest relation, has important implications for other research on circadian rhythms in health and disease, which has focused mostly on amplitude and phase. The use of the stability measures may enhance our insight into rhythm disturbances in, *e.g.*, major depression and seasonal affective disorder.

Keywords Rest-activity rhythm, Non-parametric calculations, Well-being, Dementia

EVENING ADMINISTRATION OF CAFFEINE OR MODAFINIL AVERTS THE CIRCADIAN DECLINE IN COGNITIVE PERFORMANCE AND BODY TEMPERATURE DURING SUSTAINED WAKEFULNESS

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Objectives: During sustained wakefulness cognitive performance displays a circadian rhythm with trough at dawn and elevation toward the morning. This pattern is similar in other physiological measures associated with vigilance, like blood pressure, heart rate, and body temperature. One of the principal methods of averting the nocturnal decline in cognitive performance is by administration of stimulants. The most widely used and well documented stimulant is caffeine. Another relatively new stimulant drug is Modafinil. However, the underlying mechanism of this vigilance maintenance at night is not fully understood. One possible mechanism is via elevation of body temperature. However, the data about the effect of modafinil or caffeine on body temperature during sustained wakefulness

are very limited and the results are mixed. Therefore, the aim of the present study was to examine this effect in a placebo-controlled, double-blind design.

Methods: The influence of modafinil and caffeine on the oral temperature, performance level in a flight simulation task, and subjective vigilance ratings was examined at 7 time points (2 h spaced) during a single night and following morning, *i.e.*, between 23:00 and 11:00 h. Each subject was examined under 4 conditions on 4 separate nights in mixed order: baseline (no treatment), placebo (starch, 200 mg), caffeine (200 mg), and Modafinil (200 mg), all of which were administered once, at 23:00 h.

Results: Without treatment or under placebo administration, the performance level (reaction time and accuracy), subjective vigilance ratings, and oral temperature showed significant decline at 05:00 and 07:00 h and elevation again in the morning (09:00 and 11:00 h). However, with single administration of either modafinil (200 mg) or caffeine (200 mg) at 23:00 h, the decline in all measures, namely, performance level, subjective vigilance ratings, and oral temperature was completely abolished. Yet, interestingly, the morning elevation in these measures was maintained.

Conclusions: We found that the maintenance of vigilance and cognitive performance during sustained wakefulness by Modafinil or caffeine is strongly linked to the maintenance of the body temperature level. This notion could contribute to the development of other means for vigilance management during sustained wakefulness using methods to prevent the nocturnal drop in body temperature.

Keywords Body temperature, Cognitive performance, Caffeine, Modafinil, Circadian, Sustained wakefulness

THE EFFECTS OF MILD HYPOXIA ON HUMAN CIRCADIAN TIME STRUCTURE

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Objective: Fatigue is often reported after long duration flights. Mild hypobaric hypoxia caused by pressurization may be involved in this effect by alteration of circadian time structure, even if no time zones are crossed. In this controlled crossover study we assessed the effects of two levels of

hypoxia equivalent to 8,000 and 12,000 ft on circadian rhythm in core body temperature (CBT) and plasma melatonin.

Methods: 20 healthy young male volunteers (23 to 39 yrs) were exposed in a hypobaric chamber for 8 h (08:00 to 16:00 h) to 8,000 ft, followed 4 wk later by 12,000 ft. CBT was recorded by telemetry. Blood samples were obtained under standardized conditions of sampling at 3 h intervals over two 24 h cycles (control and hypoxic exposure) and assayed for plasma melatonin.

Results: CBT rhythm expression was altered mainly at 12,000 ft, with a significant increase of mean CBT level ($p < 0.001$) and a delayed evening decline in CBT ($p < 0.05$). The nocturnal melatonin peak was significantly decreased after hypoxic exposure at both simulated altitudes ($p < 0.05$). This decrease depended on individual characteristics, *i.e.*, age, physical fitness, and sympathetic reactivity to hypoxia.

Conclusion: These findings suggest that mild hypoxia alters circadian time structure and therefore may by itself contribute, at least in part, to long-duration, post-flight fatigue, and also to the clinical disorders of jet lag in transmeridian flights.

Keywords Hypobaric hypoxia, Pressurized cabins, Circadian rhythm, Core body temperature, Plasma melatonin, Fatigue

SLEEP IMPAIRMENT IN SOME OFFSHORE SHIFTWORK SCHEDULES

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Objectives: Sleep parameters are reported as being worse in shift/night workers (Akerstedt et al., *J. Psychosomatic Res.* 2002; 53: 741–8; Ohayon et al., *J. Psychosomatic Res.* 2002; 53: 577–83) attributed partly to desynchrony of sleep period and body clock, but also to conditions of the daytime sleep environment (*e.g.*, light levels, family, and noise). The offshore oil and gas industry attempts to provide conditions that reduce daytime sleep disturbances by segregation of work and sleep areas that are sound and light insulated. However, the variety of offshore shift patterns operated may introduce variation in sleep. Our objective was therefore to identify sleep parameter differences within and between 4 offshore shift schedules.

Methods: We investigated sleep parameters (duration, latency, efficiency, and fragmentation) in male offshore shift workers ($n = 42$) using

wrist actigraphy monitors (Actiwatch-L, CNT, Cambridge, UK) in 4 schedules over 14 days: day shift of 0600 to 1800 h (DS, $n = 16$); night shift of 1800 to 0600 h (NS, $n = 11$); swing shift of 7 nights of 1800 to 0600 h and 7 days 0600 to 1800 h (7N7D, $n = 11$); and a drilling swing shift of 7 days of 1200 to 2400 h and 7 nights of 0000 to 1200 h (drill, $n = 4$).

Results: All shifts reduced sleep duration compared with onshore non-shift workers (Leger et al., *Clin. Neurophysiol.* 2002; 13(10):1607-14), which suggests there is residual effect of being 'offshore.' However, no significant overall differences in sleep parameters were found between the schedules (RM-ANOVA factors: day, schedule), suggesting that strategies implemented to reduce sleep impairment associated with daytime sleep in shift workers were successful. There was a small but significant increase in sleep efficiency over the 14 N tour duration (single factor ANOVA; $p = 0.03$; *post hoc* tests identified a difference between day 3 and day 13). This may be attributed to improved sleep as circadian resynchrony occurs, or it may reflect cumulative fatigue. In the 2 swing shift schedules, 7N7D and 'drill,' the shortest sleep duration was observed on the day of, and for one sleep episode after, the shift change-over. Further, significant increase in sleep efficiency was found after the change back to days during the 7N7D schedule ($p \leq 0.05$). Similar recovery was not seen in the 'drill' schedule after the shift change to nights. This may be because this shift change requires an advance of the sleep period, which is contrary to the natural tendency of the internal clock to delay. No significant impairment of sleep was seen on the first sleep period of the NS and 7N7D; this surprising finding may be due to a sleep debt incurred after the first night shift conferring an increase in sleep propensity.

Conclusions: We conclude that the mid-schedule changeover, which causes a sudden increase in desynchrony of the sleep period and body clock, has a negative impact on sleep duration and quality.

Keywords Shift work, Sleep, Circadian rhythm

SLEEP-WAKEFULNESS RHYTHM IN PATIENTS (PTS) WITH HEAD AND NECK CANCER

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Objective: The aim of the study was to evaluate the sleep-wakefulness rhythm in pts with head and neck cancer.

Methods: 9 pts consisting of 5 males and 4 females with head and neck cancer were chosen from the Regional Cancer Centre, Pt. J.N. Medical

College, Raipur, India. They were receiving either chemotherapy ($n = 4$), radiotherapy ($n = 3$), or both ($n = 2$) at the time of the study and wore an actiwatch (AW64, Mini Mitter Co., Inc., USA) for at least 4 days. Data collected at 0.25 min epoch intervals were retrieved and analyzed with specific actiware-sleep software. In addition, several other physiological variables, such as auxiliary temperature (AT), systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were also measured at least 6 to 8 times per day for the entire duration of study. Further, various sleep parameters, such as sleep efficiency, sleep latency, time in bed, assumed sleep, actual sleep time, actual wake time, and fragmentation index were also analyzed. Other statistical techniques, such as cosinor rhythmometry, ANOVA, and Duncan's multiple-range test were also used.

Results: A statistically significant circadian rhythm in sleep-wakefulness with a period of 24 h was observed in all pts. Results further indicate a significantly higher amplitude (as % of mesor) ($p < 0.05$) in the wrist activity of female compared to male pts. However, for other variables, namely AT, SBP, DBP, and HR, significant circadian rhythms could not be validated. In addition, female pts experienced better sleep efficiency ($p < 0.05$) and had longer actual sleep time ($p < 0.05$) compared to male pts.

Conclusion: This is the first study of its kind in India and provides meaningful data for the implementation of chronochemotherapy and chronoradiotherapy. However, further study involving more pts is desirable.

Keywords Sleep-wakefulness rhythm, Systolic blood pressure, Diastolic blood pressure, Heart rate, Head and neck cancer, Actigraphy

Supported by UGC, New Delhi under DRS-SAP; and DST, New Delhi under Fast-Track and FIST Scheme

SLEEP-WAKEFULNESS RHYTHM IN HUMAN SUBJECTS OF SUB-TROPICAL INDIA AS FUNCTION OF CHRONOTYPE AND SEX

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Objective: The aim of the study was to investigate the characteristic features of sleep-wake rhythm in human subjects of sub-tropical India as function of chronotype and sex.

Methods: 24 apparently healthy human subjects consisting of 13 males and 11 females volunteered for study. The subjects comprised of 8

morning type (MT), 9 intermediate type (IT), and 7 evening type (ET) individuals. The sleep-wake rhythm of each subject was monitored by Actiwatch® (AW64, Mini Mitter Co., Inc., USA). Data were collected with 1 min epoch for at least 4 to 8 days and analyzed using actiware-sleep software. Other statistical techniques, such as cosinor rhythmometry, ANOVA, and Duncan's multiple-range test were also used.

Results: All subjects exhibited a statistically significant circadian rhythm in sleep-wakefulness with a period very close to 24 h. The acrophase was earlier ($p < 0.05$) by at least 1 h in MT compared to ET. It also occurred earlier ($p < 0.05$) in females than males. Moreover, females experienced better sleep efficiency and shorter sleep latency than males.

Conclusion: Our findings show an association between phase of sleep-wakefulness rhythm and circadian typology. Furthermore, sleep efficiency and latency showed a sex-related association. These findings have an important bearing on the interpretation of interindividual differences in the characteristics of circadian rhythm and sleep physiology in humans.

Keywords Sleep-wakefulness, Circadian rhythm, Chronotype, Sex, Sleep efficiency, Sleep latency

Supported by UGC, New Delhi under DRS-SAP; and DST, New Delhi under Fast-Track and FIST Schemes

ORAL PRESENTATION SESSION J

CHRONOTHERAPEUTIC OPTIMIZATION OF ANTIHYPERTENSIVE THERAPY IS FOLLOWED BY BENEFICIAL CHANGES OF MORPHOLOGIC AND FUNCTIONAL ECHOCARDIOGRAPHIC PARAMETERS

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Objectives: Left ventricular hypertrophy is an independent risk factor and also a strong and independent predictor of cardiovascular morbidity and mortality. The aim of our study was too examine if a chronotherapeutic optimization of the antihypertensive effect (reaching a "dipper" status, mBP, SBP, DBP values in normal range, PTE <25%, PP <53 mm Hg, attenuation of the morning surge, absence of significant periods of hypotension) of different drugs is followed by beneficial changes of morphologic and functional echocardiographic parameters.

Methods: We studied 60 consecutive diurnally active pts with mild-to-moderate essential hypertension (by ABPM); 11 were treated with Enalapril maleat–Enap (KRKA) 20 mg/day [7 "dippers' (D) (defined by a fall from the daytime mean BP by 10 to 20% during the night), 4 "non-dippers" (ND) (fall of >10% of BP values during the night), 1 "undetermined" (UD),15 with Verapamil – Isoptin RR 240 (KNOLL) mg/day [9D, 5ND,1UD, and 34 with Amlodipine–Norvasc (Pfizer) 10 mg/day [20D, 11 ND, 2 UD]. The medications were taken as a single administration in the morning for at least 1 month; however, the results of this schedule with the mentioned doses were considered suboptimal, because of lack of significant changes in echocardiographic parameters. By replacing the morning with a vesperal administration of the mentioned drugs, in the same dosage, all the subjects became "dippers" and reached the chronotherapeutic optimization. ABPM was performed with an ABPM 04 Meditech device (with BHS and AAMI validation). We used a Hewlett-Packard Sonos 1000 device to assess echocardiographic parameters. We studied at 3 and 6 months (mo) the following parameters: IVS and posterior LV wall thickness, LVID, LV mass, LV mass index, maximal E and A wave speed, E/A, and fractional shortening, and ejection fraction. We used the χ^2 test for establishing statistical significance.

Results: Comparison of the morphologic and functional changes before and after attaining "dipper" status and optimal therapeutic results, beneficial changes with statistical significance ($p < 0.0005$) were obtained at 3 mo and confirmed also at 6 mo. There were no statistical significant differences between the demographic characteristics of the 3 groups of pts and between the therapeutic efficacy of the 3 treatment regimens. The final results (at 6 mo of surveillance) showed no statistically significant differences of the morphologic and functional parameters, compared to normotensives with the same demographic characteristics.

Conclusions: A "normalization" of the circadian BP pattern and values can be obtained by proper circadian timing of medications, which is followed by beneficial echocardiographic morphologic and functional changes.

Keywords Hypertension, Dipper hypertension, Non-dipper hypertension, Left ventricular hypertrophy, Functional left ventricular changes, Chronotherapy

CIRCADIAN VARIATIONS OF AMBULATORY PULSE PRESSURE (PP), BLOOD PRESSURE (BP) AND HEART RATE (HR) PROUDCT (RPP) IN HEALTHY, HYPERTENSIVE AND DIABETIC SUBJECTS

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Objective: PP, HR, and RPP (or double product: HR × SBP) have been considered as independent predictors of cardiovascular events and mortality in recent years. We analyzed and compared PP and RPP in healthy, hypertensive and diabetic subjects by 24 h ambulatory blood pressure monitoring (ABPM).

Methods: 20 male and 30 female healthy controls (C) aged 60.0 ± 10.3 (SD) yrs and 33 male and 25 female mild or moderate hypertensive (H) pts aged 59.4 ± 8.0 yrs; and 28 male and 19 female type 2 diabetes (D) pts, clinically normotensive without insulin treatment, aged 61.0 ± 8.5 yrs were studied. The TM-2421 ABPM (Japan, A&D) was used for 24 h BP measurement with data being analyzed by the Cosinor method for circadian rhythms.

Results: Ambulatory PP and RPP showed significant circadian rhythmicity. The mean and Mesor of the PP and RPP were highest in H, moderate in D, and lowest in C. Significant differences were found between C and H for almost all the study variables, while only the mean and Mesor of the RPP differed between the C and D. The 24 h mean the RPP and the Mesor of both the PP and RPP between the H and D.

Conclusion: Circadian variations of both ambulatory PP and RPP were confirmed in healthy, H and D subjects. The values of PP and RPP differed significantly between the study groups, suggesting changes in both H and D pts, with implications for clinical normotensive D pts.

Keywords Ambulatory blood pressure monitoring, Pulse pressure, Double product, Rate pressure product, Circadian rhythm, Hypertension, Diabetes

PERIOPERATIVE CONVENTIONAL AND RHYTHM ANALYSIS OF PATIENTS UNDERGOING ELECTIVE MITRAL VALVE REPLACEMENT (MVR)

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Objective: We compared the pre and postoperative data of blood pressure (BP) and heart rate (HR) characteristics of patients with chronic atrial fibrillation (AF) undergoing MVR.

Methods: 33 pts undergoing elective MVR were studied after all medications were discontinued 2 days before admission. Pts with previous or

current history of diabetes mellitus (DM), neurologic disorders, recent myocardial infarction, arrhythmia, low ejection fraction (<40%), and pulmonary hypertension (mean PAP <30 mm Hg) were excluded. Left atrial diameter was not significantly different among patients (43.6 ± 5 mm). History of the onset of AF was 14.2 ± 3 yrs. Pts stayed 2 days in the ICU and were then transported to wards. BP was monitored hourly for 24 h (HP, Model 68S, M1166A) the day before surgery and 2 days after in the wards. Each 24 h was divided into daytime and nighttime spans based on the wards' routine of lights off (22:00 h) and breakfast (06:00 h) times. Reference time (zero) was as the time of breakfast — morning awakening at 06:00 h. All data were analyzed by ABPM-FIT and CV-SORT separately for each pt. The significance of best fit was determined by the zero-amplitude test. Comparison of mean values was performed by paired t-test with $p < 0.05$ was considered statistically significant.

Results: 19 of 33 pts (58%) were non-dippers before surgery and 32 of the 33 pts displayed non-dipper pattern after surgery. The Peak and MESOR of systolic and diastolic BP decreased significantly postoperatively, while HR markedly increased. The double amplitude (total peak-to-trough 24 h variation) of the systolic BP and HR declined significantly, but that of diastolic BP increased postoperatively. The slope of the morning rise and evening dip in BP declined and the rate-pressure product (systolic BP × HR) increased significantly after MVR.

Conclusions: Conventional and rhythm analysis of BP and HR data demonstrated significant differences between the pre- and post-operative periods in pts who underwent MVR. These preliminary results need further complementary studies with increased number of pts in a variety of settings and with evaluation of BP and HR variability in MVR pts to provide useful information about the pathophysiology of mitral disease.

Keywords Mitral valve disease, Atrial fibrillation, Blood pressure, Heart rate, Mitral valve replacement, Circadian rhythm

DIFFERING ADMINISTRATION TIME-DEPENDENT EFFECTS OF LOW-DOSE ASPIRIN ON AMBULATORY BLOOD PRESSURE IN DIPPER (D) AND NON-DIPPER (ND) HYPERTENSIVE SUBJECTS

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Objectives: Aspirin (ASA) is a potent antioxidative agent that markedly reduces vascular production of superoxide, prevents angiotensin II-induced hypertension and cardiovascular hypertrophy, and induces nitric oxide (NO) release from vascular endothelium. Low-dose ASA has also been shown to reduce blood pressure (BP) in hypertensive pts when administered before bedtime, as opposed to upon awakening, (*Hypertension*. 2003;41:1259–1267), possibly enhancing the nocturnal trough in NO production. Since endothelium-dependent vasodilation is blunted through decrease in NO release in ND in comparison with D hypertensive pts, we investigated the administration time-dependent influence of ASA on BP in D and ND hypertensive subjects.

Methods: We studied 235 untreated pts with grade 1 hypertension (97 men), 45.3 ± 12.4 yrs of age, randomly assigned to receive ASA (100 mg/day) either upon awakening or at bedtime. BP was ambulatory monitored for 48 h at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, at baseline and after 3 months of intervention.

Results: Compared to baseline, BP was slightly elevated with ASA upon awakening, mainly in D pts and during nocturnal hours (increase of 1.4 and 1.0 mm Hg in the 24 h mean of systolic and diastolic BP; $p < 0.048$). A highly significant BP reduction was observed in pts who received ASA at bedtime (decrease of 7.0 and 4.9 mm Hg in systolic and diastolic BP; $p < 0.001$). The reduction in nocturnal BP mean was more than double in ND (11.0 and 7.0 mm Hg) compared to D (5.0 and 3.1 mm Hg; $p < 0.001$ between groups).

Conclusions: This prospective trial corroborates the highly significant administration time-dependent effect of low-dose ASA on BP, mainly in ND hypertensive pts. The timed administration of low-dose ASA could thus provide a valuable approach, beyond the secondary prevention of cardiovascular disease, in the BP control of pts with mild essential hypertension.

Keywords Hypertension, Aspirin, Chronotherapy, Non-dipper hypertension, Dipper hypertension

ADMINISTRATION TIME-DEPENDENT EFFECTS OF NIFEDIPINE GITS ON AMBULATORY BLOOD PRESSURE (BP) IN HYPERTENSIVE PATIENTS

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Objectives: Several studies have shown that the circadian pattern of BP remains unchanged after either morning or evening dosing of several calcium channel blocker (CCB) medications, including amlodipine, isradipine, verapamil, nitrendipine, and cilnidipine. We investigated the administration time-dependent antihypertensive efficacy of nifedipine GITS in doses of 30 and 60 mg/d.

Methods: We studied 80 previously untreated pts with grade 1 to 2 essential hypertension (36 men), 52.1 ± 10.7 yrs of age, randomly assigned to receive nifedipine GITS (30 mg/d) as a monotherapy for 8 weeks either upon awakening or at bedtime. Uncontrolled pts were titrated to receive 60 mg/d nifedipine GITS for another 8 wks. BP was measured by ambulatory monitoring for 48 h at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after every 8 wks of therapy.

Results: The BP reduction with the lower dose of 30 mg/d was slightly, but not significantly, larger after bedtime dosing (10.3 and 6.5 mm Hg reduction in the 24 h mean of systolic and diastolic BP with nifedipine GITS upon awakening; 12.0 and 7.2 mm Hg with bedtime dosing). In non-responders to this low dose, the efficacy of 60 mg/d nifedipine GITS was markedly larger with bedtime dosing (16.0 and 9.3 mm Hg in systolic and diastolic BP, compared to 8.4 and 4.0 mm Hg with morning dosing; $p < 0.03$). Moreover, bedtime nifedipine GITS reduced the incidence of edema by 91% and total number of secondary effects by 74% compared to morning dosing ($p = 0.020$).

Conclusions: The added efficacy at the lower dose of 30 mg/d, the marked benefits with 60 mg/d, and the safety profile of bedtime as compared to morning administration of nifedipine GITS should be taken into account when prescribing this CCB in the treatment of essential hypertension.

Keywords Hypertension, Nifedipine GITS, Chronotherapy, Circadian rhythm, Calcium channel blocker medications

ADMINISTRATION TIME-DEPENDENT EFFECTS OF TORASEMIDE ON AMBULATORY BLOOD PRESSURE (BP) IN HYPRTENSIVE PATIENTS

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Objectives: Torasemide (T) is a high ceiling loop diuretic frequently used to treat congestive heart failure and hypertension (*Drug Saf.* 1996;14:104–120). Diuretics have not yet been studied in relation to possible circadian variation of effects. Accordingly, we investigated the administration time-dependent efficacy of T in hypertensive pts.

Methods: We studied 58 previously untreated pts with grade 1 to 2 essential hypertension (25 men), 48.7 ± 11.9 yrs of age, randomly assigned to receive T (5 mg/day) as a monotherapy either upon awakening or at bedtime. BP was measured for 48 h by ambulatory monitoring at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after 6 wks of therapy.

Results: Efficacy of T was significantly higher with bedtime dosing (11.2 and 8.0 mm Hg reduction in the 24 h mean systolic and diastolic BP, respectively) compared to the drug ingestion upon awakening (6.2 and 3.7 mm Hg reduction in systolic and diastolic BP). The percentage of pts with controlled ambulatory BP after treatment was also higher with bedtime treatment (52 *vs.* 28%; $p = 0.042$). The time-response curves indicate a full 24 h therapeutic coverage *only* when T was administered before bedtime. The 24 h urinary secretion of sodium and potassium remained unchanged after treatment in both dosing-time groups.

Conclusions: A single daily dose of 5 mg/day T is effective for BP reduction mainly with bedtime administration. The differences in efficacy and therapeutic coverage as a function of the circadian time of T ingestion as documented in our study should be taken into account when prescribing this loop diuretic for the treatment of pts with essential hypertension.

Keywords Hypertension, Torasemide, Circadian rhythm, Chronotherapy, Diuretic medication

CHRONOTHERAPY IMPROVES BLOOD PRESSURE (BP) CONTROL AND REVERTS THE NON-DIPPER PATTERN IN PATIENTS WITH RESISTANT HYPERTENSION (RH)

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Objectives: Therapeutic strategies for RH currently include adding additional or changing medications in search for a better synergic combination. Most pts, however, take all their drugs at a single time of day, in the

morning (*J. Hypertens.* 2002;20:1097–1104). We evaluated the impact of modifying the time of treatment, without increasing the number of prescribed medications, on the circadian BP pattern.

Methods: We studied 147 pts with RH (79 men), 60.1 ± 12.0 yrs of age, who were prescribed 3 antihypertensive drugs and were ingesting all them at a single time of day—in the morning, Pts were randomly assigned to one of two groups according to the modification in their treatment strategy: (1) one of the medications was changed, but keeping all 3 continued to be ingested in the morning; (2) the same approach as in (1) but the changed medication was taken at bedtime. BP was measured for 48 h ABPM at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, at baseline and after 3 months (mo) of treatment with the new therapeutic scheme.

Results: There was no additional therapeutic effect on BP when all medications continued to be taken in the morning upon awakening (0.5 and 0.7 mm Hg reduction in the 24 h mean of systolic and diastolic BP; $p > 0.374$). At baseline, only 22% of the pts in this group were dippers (D), and this percentage was further reduced to 15% after 3 mo therapy with all drugs upon awakening. The BP reduction was statistically significant (8.6 and 5.9 mm Hg for systolic and diastolic BP; $p < 0.001$) when 1 of the 3 medications was ingested at bedtime. This effect was markedly larger on the nocturnal mean of BP. Thus, while only 13% of the pts in this group were D at baseline, 52% were converted to D after 3 mos of this therapeutic regimen.

Conclusions: Results of this prospective trial on RH pts indicate that that time of treatment may be more important for BP control and for the proper modeling of the circadian BP pattern than the combination of medications *per se*.

Keywords Resistant hypertension, Chronotherapy, Circadian rhythm, Dipper hypertension, Non-dipper hypertension

TREATMENT OF NON-DIPPER (ND) ESSENTIAL HYPERTENSION WITH BEDTIME ADMINISTRATION OF VALSARTAN (V)

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Objectives: Previous results have indicated that ingestion of V at bedtime, as opposed to upon wakening, improves the day/night ratio of

blood pressure (BP) towards a more dipper (D) pattern without loss in 24 h efficacy (*Hypertension*. 2003;42:283–290). Some studies have found differential effects of antihypertensive drugs according to the D status of pts. Accordingly, we investigated the administration time-dependent antihypertensive efficacy of V in ND pts.

Methods: We studied 190 ND pts with grade 1 to 2 essential hypertension (72 men), 53.9 ± 13.2 yrs of age, randomly assigned to receive single daily V monotherapy (160 mg/d) either upon awakening or at bedtime. BP was measured by ABPM for 48 h at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after 3 months (mo) of therapy.

Results: The highly significant BP reduction after treatment ($p < 0.001$) was slightly larger with bedtime V dosing (13.0 and 8.1 mm Hg reduction in the 24 h mean of systolic and diastolic BP with V taken upon awakening; 15.2 and 10.6 mm Hg with V taken at bedtime). The day/night BP ratio was unchanged with V ingestion upon awakening (0.4 and 0.7 for systolic and diastolic BP; $p > 0.309$). This ratio was significantly increased (7.2 and 7.1 for systolic and diastolic BP; $p < 0.001$) when V was taken at bedtime, which resulted in 75% of the pts reverting to D, a significant increase in the proportion of pts with controlled BP, and significant reduction in urinary albumin excretion.

Conclusions: In hypertensive ND pts, V ingestion at bedtime increases drug efficacy during the nocturnal resting hours, improves renal function, and potentially reduces cardiovascular risk.

Keywords Hypertension, Valsartan, Chronotherapy, Non-dippers hypertension

ADMINISTRATION TIME-DEPENDENT EFFECTS OF VALSARTAN (V) ON URINARY ALBUMIN EXCRETION IN (UAE) HYPERTENSIVE SUBJECTS

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Objectives: Results of previous studies indicate V administration at bedtime, as opposed to upon wakening, improves the diurnal/nocturnal ratio of blood pressure (BP) towards a more dipper (D) pattern without loss in 24 h efficacy (*Hypertension* 2003;42:283–290). UAE in non-dippers (ND) has been shown to be significantly greater than in D hypertension

(*Am J Hypertens.* 1994;7:23–29). We hypothesized that improving the diurnal/nocturnal BP ratio could further reduce UAE in hypertensive pts.

Methods: We studied 123 previously untreated non-proteinuric pts with grade 1-2 essential hypertension (50 men), 51.0 ± 11.4 yrs of age, randomly assigned to receive V (160 mg/day) as a monotherapy, either upon awakening or at bedtime. BP was measured for 48 h by ambulatory monitoring at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after 3 months (mo) of therapy.

Results: The significant BP reduction after 3 mo of V dosing was similar for both treatment times (13.9 and 9.1 mm Hg reduction in the 24 h mean of systolic and diastolic BP with V taken upon awakening; 15.2 and 10.3 mm Hg with V taken at bedtime). The diurnal/nocturnal BP ratio was unchanged after V ingestion on awakening, but significantly increased by 5.4% when taken before bedtime. UAE was significantly reduced, mainly after bedtime treatment (31% reduction). This reduction was independent of the 24 h BP decrease, but highly correlated with the decrease of nocturnal BP ($r = 0.326$; $p = 0.007$) and with the increase in diurnal/nocturnal ratio ($r = 0.395$; $p = 0.002$).

Conclusions: The bedtime V treatment schedule improves the diurnal/nocturnal BP ratio to a more dipper profile. This results in further decrease of UAE compared to the morning treatment schedule, and could thus reduce cardiovascular risk.

Keywords Hypertension, Dipper hypertension, Valsartan, Albumin excretion, Chronotherapy

ADMINISTRATION TIME-DEPENDENT EFFICACY OF VALSARTAN (V)-ATORVASTATIN (A) COMBINATION IN HYPERLIPIDEMIC PATIENTS (PTS) WITH ESSENTIAL HYPERTENSION

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Objectives: Nighttime dosing of A has an added capacity to reduce blood pressure (BP) beyond that expected based on lipid reduction. Moreover, V ingestion at bedtime, as opposed to upon wakening, improves the day/night BP ratio towards a more dipper pattern, without loss in 24 h efficacy (*Hypertension* 2003;42:283–290). Accordingly, we have evaluated the

potential administration time-dependent synergic effects of V and A taken in combination.

Methods: We studied 80 (32 men), 56.3 ± 9.3 yrs of age, grade 1 to 2 essential hypertensive pts with primary hypercholesterolemia. Pts were randomly assigned to receive single daily V monotherapy (160 mg/d) either upon awakening or at bedtime. Half of the pts also received 10 mg/d A, always at bedtime. BP was measured for 48 h by ABPM at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after 3 months (mo) of therapy.

Results: Efficacy of V was significantly improved when combined with A (13.9 and 9.6 mm Hg reduction in the 24 h mean of systolic and diastolic BP with V antihypertensive monotherapy; 17.1 and 10.7 mm Hg reduction with V combined with A; $p < 0.027$ between groups). V, in combination with A, was equally effective when administered either upon awakening or at bedtime in reducing the diurnal BP mean ($p = 0.620$). However, bedtime V dosing significantly improved efficacy on the nocturnal BP mean (11.0 and 6.8 mm Hg reduction in systolic and diastolic BP with morning dosing; 23.5 and 15.9 mm Hg with bedtime dosing; $p < 0.001$). This resulted in a significant increase in the day/night BP ratio to a more dipper profile (5.5 and 8.0 in systolic and diastolic BP ratio; $p < 0.001$).

Conclusions: A significantly enhances the antihypertensive efficacy of V. This synergic combination markedly improves control of nocturnal BP, without loss in 24 h efficacy, when both drugs are taken at bedtime.

Keywords Hypertension, Valsartan, Atorvastatin, Chronotherapy, Day/night blood pressure ration, Dipper hypertension

SYMPOSIUM IX-CHRONOBIOLOGIC OPTIMIZATION OF MEDICAL TREATMENTS IN WOMEN

GENDER ISSUES IN THE EPIDEMIOLOGY OF CARDIOVASCULAR DISEASE

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A growing body of evidence substantiates that the occurrence of cardiovascular events is unevenly distributed in time, especially during the 24 h. These temporal patterns are indicative of temporal variation in the: (1) pathophysiological mechanisms that trigger cardiovascular events and

(2) physiological status of the cardiovascular system, which combine to give rise to 24 h and other periodicities in the *susceptibility* to disease. The classic assumption of epidemiologic studies is constancy (or homeostasis) in risk to disease during the 24 h, as well as other time domains. However, a new concept seems more appropriate, that of *chronorisk* since it takes into account the temporal variability in the pathophysiological mechanisms and their reciprocal temporal interactions that lead to day-night and other time-dependent patterns in cardiovascular events. This chronobiological approach, which is expected to contribute new insight into the prognostic and therapeutic assessment of cardiovascular events, is worthy of broader application in cardiovascular and other fields of medicine and warrants further investigation. The role of 24 h and other rhythms to health, disease, and treatment of women has been little studied. The symptom intensity and risk of a variety of cardiovascular diseases is rhythmic in women as it is in men. Improving the safety, efficacy, and preventive qualities of medications in women as well as in men requires a better understanding of such fluctuations.

Keywords Gender, Cardiovascular disease, Chronorisk

LOW-DOSE ASPIRIN (LDA) CHRONOPREVENTION OF PREECLAMPSIA AND GESTATIONAL HYPERTENSION IN HIGH RISK PREGNANCIES

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In a recent prospective study we investigated the potential influence of low-dose aspirin on blood pressure and perinatal outcome in pregnant women who were at a higher risk of developing preeclampsia than the general obstetric population and who received LDA at different times of the day according to their rest-activity cycle. A double-blind, randomized, controlled trial was involved 341 pregnant women (181 primipara) randomly assigned to 1 of 6 possible groups according to type (placebo or aspirin, 100 mg/d, starting at 12 to 16 wks gestation) and time of treatment: upon awakening (time 1), 8 h after awakening (time 2), or before bedtime (time 3). Blood pressure (BP) was automatically monitored for 48 h every 4 weeks from the day of recruitment until delivery, as well as at puerperium. Compared with placebo, there was no effect of LDA on BP when taken at time 1. A BP reduction was highly statistically significant when LDA was taken daily at time 2 and, to a greater extent, at time 3

(mean reductions of 9.7/6.5 mm Hg in 24 h mean of systolic/diastolic BP at the time of delivery compared to placebo given at bedtime). Differences in BP among women taking LDA at different circadian times disappeared at puerperium ($p > 0.096$). Companion data further indicated a significant ($p = 0.006$) 71% reduction in the incidence of gestational hypertension, preeclampsia, IUGR, and preterm delivery in groups taking LDA at time 2 and time 3, compared to the other 4 groups, where treatment had no BP effect. Results thus indicate a highly significant effect of LDA on BP that is markedly dependent on the time of its ingestion with respect to the rest-activity cycle. The use of doses of aspirin <80 mg/day that do not affect placental thromboxane, initiation of the use of aspirin after 16 weeks of gestation, and the lack of circadian timing for aspirin administration, could all explain the lack of positive results in many previous clinical trials. Timed use of LDA effectively contributes to BP control in women who at high risk of preeclampsia.

Keywords Low dose aspirin, Gestational hypertension, Chronotherapy, Pregnancy complications

SEXUAL DIMORPHISMS, MENSTRUAL CYCLES, AND SEASONS: CHRONOBIOLOGY OF WOMEN'S CANCER HEALTH

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Sex, menstrual cycles, and seasons affect the host-cancer balance, the efficiency of cancer diagnosis, and cancer treatment efficacy: (1) Cancer is a sexually dimorphic malady. Female sex is associated with superior outcomes for every cancer shared by members of both sexes. For childhood cancer, female sex advantage does not appear until menarche; and for cancers of adults, diminishes after menopause. In adults, the advantage of being female increases with decreasing median age of diagnosis for each particular cancer. For example, the benefit of being female for colorectal cancer, with a median age at diagnosis of 70, is significant, but several-fold smaller than for melanoma with a median age of 50. The first line treatment for most, if not all, of these cancers is curative surgical resection. Therefore, the relationship among cancer, surgery, and sex is apparently important. (2) The menopausal status of women screened for breast cancer (BC) determines the utility of screening mammography. Postmenopausal women benefit from mammographic screening, which lowers their BC mortality. Across 8 large randomized controlled clinical trials of screening

mammography, it was found that women under age 50 yrs do not benefit from screening mammography. In fact, their BC mortality is increased for the first 8 yrs of follow-up compared to unscreened premenopausal women. This disadvantage of screening occurs most prominently in young women with node-positive BC. Tumor dormancy is adversely affected selectively among node-positive premenopausal women by the surgical wounding associated with breast biopsies and BC resections, incited by abnormalities discovered by screening mammography. (3) Among premenopausal women, the menstrual cycle, *per se*, is also relevant to the signal-to-noise ratio of BC screening. Twice as many BC are missed in women who undergo screening mammography during the luteal phase, near to and 7 to 14 days following putative monthly ovulation, than when this radiographic procedure performed in the follicular phase, near to and following monthly menses. (4) The timing of BC resection within the premenopausal woman's menstrual cycle is relevant to whether the young woman with node-positive BC is cured by the operation and subsequent treatments, or she ultimately succumbs to metastatic BC. Those women resected near to and 7 to 10 days following putative ovulation have a 25% better chance of 10 yr disease-free survival than those resected near to and following menses. The beneficial effect of optimal breast cancer resection timing is, on average, 2 to 3 times greater than the salutary effect of adjuvant chemotherapy. These outcome effects are mediated by sex hormone-dependent, wounding-mediated, menstrual cycle stage-dependent, changes in VEGF/bFGF ratio, capillary permeability, tumor cell DNA synthesis, mitotic index, and metastatic potential. (5) The menstrual/estrous cycle phase of chemotherapy modulates the toxicities of cytotoxic drugs. The timing of 5-FU within the fertility cycle determines the amount of bone marrow and gut tissue injury, and subsequent recovery of reproductive function (fertility and fecundity). Reproductive and other toxicities can be minimized by luteal phase timed chemotherapy treatment. This is potentially important for young women who need to undergo adjuvant chemotherapy to increase their chances of cure, and who still want to have children. (6) Seasons also affect BC biology. BC is most commonly diagnosed in spring. BC estrogen and progesterone receptor positivity is least likely in spring. BV histopathology is most aggressive in spring and the majority of premenopausal BC deaths occur during spring each year. (7) Cervical cancer (CC) screening results are affected by season. Inspection of a million Pap smears over 16 consecutive yrs reveals that the histological footprints of HPV infection, all grades of cervical dysplasia, as well as all grades of cervical epithelial cancer are twice as common in summer (August) than other seasons. Since most (4/5) such abnormalities resolve spontaneously, and since 3 to 4 logs more women are told they have an actionable Pap smear abnormality than die from CC, among regularly screened populations, the

signal-to-noise ratio and the medical meaning of the cervical epithelial abnormality may be different, depending upon the season of Pap smear screening. This chronobiology has clinically meaningful and important individual and public health implications. The seasonality of these changes in the cervical epithelium correlate with the seasonality of population exposure to the immunosuppressive and virus activating effects of sunlight and its UVB component, and may, thereby, be behaviorally avoidable. (8) In summary, the reproductive life cycle stage, menstrual cycle stage, and annual cycle stage/season of cancer screening, diagnosis, and treatment, each impact cancer outcome in women. Failure to take these low frequency biologic rhythms into careful consideration will make it hard, if not impossible, to maximize the healthcare benefits of cancer screening and treatment among women.

Keywords Gender, Women, Cancer screening, Cancer treatment, Cancer outcomes, Circadian rhythm, Menstrual cycle, Season, Life stage

GENDER-RELATED SEASONAL PATTERNS AND TREATMENT OF AFFECTIVE DISORDERS

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Women are overrepresented at a 2:1 to 4:1 ratio among pts with winter depression, or seasonal affective disorder (SAD). SAD is a subtype of major depression characterized by recurrent fall/winter episodes of depression and spontaneous remission of symptoms in spring/summer. Circadian rhythm and neurotransmitter hypotheses have been prominent in explaining the pathophysiology of SAD and winter dysphoria. SAD is also associated with so-called atypical features of depression, including overeating, weight gain, oversleeping, and extreme fatigue. The clinical features of SAD are also found in other gender-based psychiatric conditions, such as the eating disorder, bulimia nervosa, and premenstrual depressive disorder (PMDD). It has long been recognized that there is substantial overlap between depression and these disorders. However, more recent investigations of bulimia and PMDD have also demonstrated marked seasonal symptom patterns. Up to 1/3 of women with these conditions experience significant winter worsening of mood and other symptoms to a degree similar to that seen in SAD. Bright light therapy is an effective treatment for SAD, and preliminary studies in bulimia and PMDD also show its beneficial effects on mood and other core symptoms. SAD, bulimia, and PMDD may be related via a common pathophysiology

resulting from seasonal changes in brain serotonin or dopamine function. Recent genetic studies show that polymorphisms in the dopamine DRD4 gene may be associated with several of these gender-specific conditions, mediated through seasonal changes in binge-eating behavior. From an evolutionary point of view, it is possible that these findings indicate an evolutionary advantage for seasonal weight gain in women that is no longer necessary in modern times, leading to pathological symptoms. Further study of the seasonal aspects of these gender-specific conditions will yield important information for diagnostic, treatment, and pathophysiological perspectives.

Keywords Seasonality, Affective disorder, Bulimia, Premenstrual depression, Antidepressants, Women, Gender

SYMPOSIUM X-MELATONIN: HORMONE AND DRUG

MELATONIN RECEPTORS IN HUMANS: BIOLOGICAL ROLE AND CLINICAL RELEVANCE

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In addition to its antioxidative effects, melatonin acts through specific membrane and nuclear receptors. To date, 2 G-protein coupled melatonin membrane receptors, MT_1 and MT_2, have been cloned in mammals, while the newly purified MT_3 protein belongs to the family of quinone reductases. Screening studies have shown that various tissues of rodents express MT_1 and/or MT_2 melatonin receptors. In humans, melatonin receptors were also detected in several cells, tissues, and organs, including brain and retina, cardiovascular system, liver, and gallbladder, intestine, kidney, immune cells, adipocytes, prostate and breast epithelial cells, ovary/granulosa cells, myometrium, and skin. The role of melatonin in human physiology is still under investigation. There is growing evidence that melatonin is involved in the regulation of circadian and seasonal rhythms. Furthermore, melatonin administration is followed by a decrease in core body temperature and an increase in distal skin temperature, leading to increased heat loss. Similar temperature changes can also be found at sleep onset, suggesting a connection between melatonin-mediated themoregulatory effects and sleep. The hypnotic effects of administered melatonin have been extensively documented and low nighttime melatonin levels are associated with some types of insomnia. Additional

effects of exogenous melatonin in humans are for example: (1) suppression of LH secretion, (2) reduction of systolic and diastolic blood pressure and blood norepinephrine levels, and (3) stimulation of T-helper cell activity and interleukin production. Furthermore, *in vitro* studies have shown that melatonin stimulates HCO_3^- secretion in human duodenal enterocytes, exerts oncostatic actions on human breast and prostate cancer cells, and decreases the expression of GLUT4 transporter in human adipocytes. Considering the multiple localizations of melatonin receptors in the human body, it is very likely that many of the functions mentioned above are mediated through these receptors. However, other mechanism pathways (*e.g.*, nuclear receptors, antioxidative functions, other indirect effects) also are possibly involved. The clinical relevance of knowledge of human melatonin receptors may be threefold: (1) physiologically based melatonin therapy in case of impaired melatonin production/secretion, (2) administration of melatonin in pharmacological doses for the treatment of various diseases, and (3) identification of mutations of the melatonin receptor gene in diseases which may potentially be associated with melatonin. Various diseases, such as for example coronary heart disease, sepsis, fibromyalgia, cancer, alcoholism, Alzheimer-type dementia, and primary insomnia, are associated with impaired melatonin secretion. Future work will provide more information about the clinical relevance of these findings, especially in regard to the applicability of melatonin as a drug for various medical conditions.

Keywords Melatonin receptors, Melatonin treatment, Core body temperature, Sleep, Sleep disorders, Immunity, Oncostatsis

MELATONIN RELATIONSHIPS WITH AGING

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Melatonin is the main hormone secreted by the pineal gland. It exhibits a high amplitude circadian rhythm with low levels during the day and high levels at night. The circadian rhythmicity continues into old age and is found in elderly human subjects, regardless of season. A large number of scientific papers have reported that plasma melatonin concentration declines with age in humans, with a rate in the nocturnal decrease of about 50%. However, this decline was not found in subjects following a constant routine. Beneficial effects of melatonin treatment include attenuation of delayed and advanced sleep phase syndromes, and in certain

situations entrainment of desynchronized rhythms *e.g.*, in jet lag, shift work, and aging. Melatonin has also been shown to possess anti oxidative properties. Clinical evaluation on a large scale is needed to assess the beneficial effects of melatonin in humans.

Keywords Melatonin, Aging, Sleep, Rhythm desynchronization

CURRENT UNDERSTANDING OF THE ROLE OF MELATONIN IN MALIGNANCY

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In this review the role of the pineal hormone melatonin in malignancy will be defined on the basis of current experimental, clinical, and epidemiological findings. Experimental *in vivo* and *in vitro* studies show that melatonin mainly inhibits the growth of well-differentiated tumors involving both direct cellular and indirect systemic mechanisms. In addition, melatonin possesses anti-carcinogenic properties. Therapeutic clinical studies have been limited but indicate that pts with advanced or terminal malignancies can benefit from melatonin treatment, by prolonged survival and improved quality of life. Recent epidemiological studies reveal elevated cancer risk rates among aircrew members as well as nurses on yr-long night shift routines; whereas, blind people show less malignancies than sighted individuals. These findings are currently interpreted as evidence for a central functional involvement of circadian pineal melatonin secretion in the endogenous defense processes against cancer. The validity of this hypothesis has to be discussed critically and tested stringently on the background of recent findings regarding non-visual retinal mechanisms involved in the regulation of pineal melatonin secretion by light as well as the stimulation of tumor growth in mice submitted to either repeated simulated jet lag or the surgical removal of the suprachiasmatic nuclei which, among others, generate the circadian rhythm of pineal melatonin. If melatonin does indeed prove to play the postulated central role in endogenous cancer protective mechanisms, this would open new perspectives and fully justify currently growing concerns of the potentially harmful effects of artificial light at night in homes and urban areas on the facilitation of the growth and development of human cancers. In addition, other accepted cancer-enhancing life-style habits, such as elevated alcohol consumption, may have to be evaluated for potentially negative effects on

pineal function. Finally, it will have to be considered whether substitution melatonin therapies as well as re-stimulation of pineal function can be utilized to foster endogenous defense processes against cancer.

Keywords Melatonin, Cancer, Long-distance flight, Night-shift work, Artificial light

MELATONIN (M) AND MENTAL DISORDERS

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Objectives: Studies on M in psychiatric and mood disorders, such as schizophrenia, depression or pervasive developmental disorders, have reported abnormalities in nocturnal serum levels and the urinary excretion of M or its principle metabolite, 6-sulfatoxymelatonin. Many studies in autistic disorder report sleep problems and altered circadian rhythms, such as reduced total sleep, longer sleep latency as well as nocturnal and early morning awakenings, suggesting abnormalities in M physiology. Additionally, M is of special interest in autistic disorder given reported alterations in central and peripheral serotonin neurobiology and the important role of serotonin in neurodevelopment. The objective of this research was to study nocturnal M urinary excretion in autistic disorder and the possible relationships between M levels and severity of the autistic disorder.

Methods: Nocturnal urinary excretion of 6-sulphatoxymelatonin was measured by radioimmunoassay in groups of children and adolescents with autistic disorder ($n = 49$) and normal control individuals ($n = 88$) matched on age, sex, and Tanner stage of puberty.

Results: Nocturnal 6-sulphatoxymelatonin excretion rate was significantly and substantially lower in pts with autism than in normal controls (mean $\pm$ SEM, 0.75 ± 0.11 *vs.* $1.80 \pm 0.17\,\mu g/hr$; $p = 0.0001$), and was significantly negatively correlated with severity of autistic impairments in verbal communication and play ($p < 0.05$).

Conclusions: These findings indicate clearly the nocturnal production of M is reduced in autism. Further research is warranted in order to

understand the mechanisms underlying the reduced M production, to assess the impact of altered M on the pathophysiology and behavioral expression of autistic disorder, and to determine the utility of M administration in autism. The implications of these results for future studies on M in schizophrenia and depression will be also discussed.

Keywords 6-Sulphatoxymelatonin, Melatonin, Autistic disorder, Pineal, Circadian sleep-wake rhythm

MELATONIN (M) AS A DRUG

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M is secreted at night by the pineal gland under normal environmental conditions. Although functions of this hormone are mainly based on correlative observations, there is some evidence that M stabilizes and reinforces circadian rhythms, especially temperature and sleep-wake rhythms, and strengthens their coupling. The existence of a phase-response curve of endogenous M secretion to the administration of exogenous M is the rationale for the treatment of circadian rhythm sleep disorders, plus those which occur with jetlag, blindness, and shift-work. M treatment can facilitate sleep initiation and improve altered overnight sleep. The sleep-promoting effects, however, are different from common hypnotics and are not associated with alterations in sleep architecture. Rather, M displays soporific properties. Recent meta-analyses conclude that M administration might be useful for treating insomnia, particularly in aged individuals with nocturnal deficiency or with an abnormal M pattern (delayed sleep phase syndrome). Further, studies in strictly controlled conditions and on large samples are recommended. The formulation (fast and/or sustained-release preparation since M displays a very fast turn-over) requires optimization as does the dose, timing, and duration of treatment. Finally, M administration could be of interest in situations where the circadian organization of physiological functions (for instance immune and antioxidative defenses) which depend on the M signal is disturbed. In the absence of short-term toxicity, the safety of long-term M use remains to be evaluated.

Keywords Melatonin, Treatment, Sleep wake cycle disorder, Humans

SYMPOSIUM XI-CIRCADIAN RHYTHMS IN DEMENTIA

CIRCADIAN RHYTHM DISTURBANCES IN NEURODEGENERATIVE DEMENTIA

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Background: Alzheimer disease (AD) pts typically exhibit erratic patterns of sleep and wakefulness that may involve disruption of central circadian and other sleep-/wake-related systems. We have performed a study to examine the disruption of the suprachiasmatic nucleus (SCN) and the perifornical hypothalamus orexin/hypocretin system to examine the role of neurodegeneration in sleep and circadian disturbance in Alzheimer's disease.

Methods: 19 cases, previously studied with 72 h recordings of core body temperature and locomotor activity, given a neuropathological diagnosis of AD, were selected for study. A 200 micron region on either side of the center of the dorsal SCN was sampled by staining 5 series of 10 micron sections with anti-VP (vasopressin) antibody, and anti-NT (neurotensin) antibody. To assess the degree of VP and NT neuronal loss within the SCN, 10 random fields in the dorsal SCN were photographed at 400X magnification, digitized for image analysis, and counted to determine the number of VP and NT per 0.1 mm^2 microscopic field, a measurement of cell density. Fresh tissue sections 50 microns in thickness (2 AD, 2 normal) were obtained from the Rhode Island Hospital Brain Bank and pretreated with 1% H_2O_2 and 50% methanol diluted in distilled water for 5 min. Non-specific binding sites were blocked by incubation for 1 h in normal goat serum. Sections were incubated in primary antibody and then subjected to a modified ABC technique using the Vectastain Elite ABC Rabbit Peroxidase system (Vector) with diaminobenzidine (DAB) as the chromagen (Vector). Tissue sections were mounted on glass slides with 0.1% gelatin then air-dried and cover slipped. Frozen autopsy samples of supramamillary perifornical hypothalamus (24 normal, 24 AD) were obtained from the McLean Hospital Brain Bank and used for the peptide extraction and enzyme-linked immunoassay (ELISA) of orexin concentration. Final orexin A levels were expressed as a ratio of ng orexin A/mg total protein concentration.

Results: Significant relationships between the loss of specific neuronal subpopulations in the SCN and circadian variables were observed, although no relationship with overall SCN neuronal cell loss or gliosis was observed. Orexin neuron populations in the human hypothalamus were observed in 2 discrete locations, the mid infundibular region

(roughly analogous to the rodent arcuate nucleus) and posterior hypothalamic, perifornical region. Levels of perifornical orexin/hypocretin as measured by ELISA were significantly lower in the AD cases compared to the normal controls.

Conclusions: These results suggest that overall neuronal loss is not sufficient to explain the sleep and circadian disturbances experienced by AD pts. Instead, losses in specific neuronal subpopulations appear to drive these disturbances, both within and outside of the SCN.

Keywords Alzheimer's disease, Suprachiasmatic nucleus, Sleep-wake cycle, Orexin neuron population

Supported by R01 AG20654, R01 AG009301, P50 AG13846 and the Dept. of Veterans Affairs

SHORT-TERM SUPPLEMENTATION OF BRIGHT LIGHT (L) AND MELATONIN (M): EFFECTS ON RHYTHMS AND BEHAVIOR IN PATIENTS WITH DEMENTIA

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Background: Sleep in the nursing home environment is extremely fragmented, possibly in part as a result of decreased light exposure. We present results from a series of studies that examined the effect of BL and M on sleep and circadian activity rhythms in pts with probable or possible Alzheimer's disease (AD).

Method: Over 10 yrs, over 150 institutionalized pts with severe dementia (92 with AD) were studied in 2 separate research protocols. All had 3 days of baseline recordings, 10 days of treatment, and 5 days of post-treatment follow-up. Sleep/wake activity was recorded for the continuous time with an Actillume (Ambulatory Monitoring, Inc). Treatment consisted of morning BL (>2500 Lx from 09:30 to 11:30 h), evening BL (>2500 Lx from 17:30 to 19:30 h), evening dim red light (<300 Lx from 17:30 to 19:30 h), or daytime sleep restriction. 41 pts participated in a M arm that began at least one month after the end of the BL study. Patients were randomized into either placebo or 10 mg of M (8.5 mg immediate release and 1.5 mg sustained release) which was administered by nursing staff at 22:00 h. Traditional sleep variables were computed: total sleep time (TST), wake after sleep onset (WASO), and sleep efficiency (SE). In addition, bouts of sleep and wake were tallied to examine whether sleep

was consolidated. Circadian activity rhythm was measured by a 5-parameter extended cosine model (amplitude, acrophase, nadir, slope of the curve, and relative width of the peak and trough).

Results: In general, pts exposed to higher average illumination levels during the day had more consolidated sleep at night, even when level of dementia was included in the model ($F_{2,63} = 3.36$; $p = 0.041$). Results showed that both morning and evening BL resulted in more consolidated sleep at night. The longest sleep bout increased from 65 to 88 min ($p < 0.05$) in the morning BL group and from 71 to 95 min ($p < 0.02$) in the evening BL group. Evening BL ($p < 0.01$) also increased the quality of the circadian activity rhythm and there was a trend for morning light ($p < 0.06$) to also increase the quality of the rhythm. M, on the other hand, had no effect of any measures of sleep or circadian rhythms.

Conclusions: Results of BL treatment studies suggest that morning and evening BL both consolidate sleep at night and make the circadian rhythm more robust. Therefore, increasing light exposure throughout the day and evening is likely to have the most beneficial effect on sleep and on circadian rhythms in dementia pts. Surprisingly, M administration did not lead to any improvements in sleep quantity or quality. This pt sample was severely demented, so there may have been a reduced ability of the pineal gland to respond to exogenous M. M treatment may be more effective in pts with less severe dementia. Overall, these results support the use of BL, but not M, therapy for improving sleep in patients with severe AD.

Keywords Alzheimer's disease, Dementia, Bright light, Melatonin, Therapy, Sleep, Circadian rhythm

Supported by NIA AG08415, M01 RR00827, P50 AG05131 and the Research Service of the Veterans Affairs San Diego Healthcare System

LONG-TERM SUPPLEMENTATION OF LIGHT (BL) AND MELATONIN (M): EFFECTS ON THE SLEEP-ACTIVITY RHYTHM AND COGNITION

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We present here the data of the first long-term (3.5 yrs) randomized placebo-controlled study on the efficacy of L and/or M in the treatment and prevention of sleep disturbances and, by doing so, to ameliorate

disturbances in cognition in demented elderly ($n = 189$). Stimulating the biological clock, the main regulator of the sleep-wake rhythm, by its natural time cues of BL and M seems a rational way to improve sleep, without the side effects of traditional hypnotics like benzodiazepines. Unique in this study is that we used a novel and verified effective placebo-control condition for BL treatment. Furthermore, our idea to apply ceiling mounted indirect BL turned out to be a highly convenient and feasible way for long-term application of daily BL therapy in this particular group of subjects. We found that both BL and M improved the rest-activity rhythm and sleep. In the verum-treated subjects cognitive decline was significantly attenuated. Statistical modeling strongly suggests that this attenuation was mediated by the BL-induced improvement of the rest-activity rhythm. Following up on the valuable work of many research groups suggesting such efficacy based on suboptimal controlled studies of limited size and duration (reviewed and discussed in: Van Someren et al., (2002). Functional plasticity of the circadian timing system in old age: light exposure. Prog. Brain Res. 138:205–231 and Riemersma et al., (2004). Melatonin rhythms, melatonin supplementation and sleep in old age. In *The Neuroendocrine Immune Network in Ageing, NeuroImmune Biology*, Straub RH, Mocchegiani E (eds.), pp. 195–211, Elsevier, Amsterdam), our long-term randomized placebo-controlled trial finally paves the way for the clinical application of this feasible and effective use of BL and M in the treatment and prevention of sleep-wake rhythm disturbances and its consequences in demented elderly.

Keywords Demented elderly, Bright light, Melatonin, Treatment, Sleep-wake cycle disturbances, Placebo-control protocol

ENVIRONMENTAL CONDITIONS, SLEEP, AND CIRCADIAN RHYTHMS IN THE NURSING HOME SETTING

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Objectives: Research shows environmental conditions impact circadian rhythms and sleep/wake patterns among older people in nursing homes. Two important environmental factors are daytime illumination levels and sleep-disruptive noises during the night. Here we describe results from 3 studies in which daytime light conditions and patient (pt) light exposure levels were recorded and nighttime noises in pt rooms

were measured. We also discuss the association between these environmental factors and sleep/wake patterns in these settings.

Methods: 378 older (age >65 yrs) long-stay and short-stay (rehabilitation) nursing home pts were studied for a total of over 2,000 nights. Participants were studied using wrist actigraphy (to estimate sleep/wake patterns and circadian activity rhythms) and bedside monitors were used to assess noise (decibels, dB) and light levels (Lx). Data were divided into "day" (08:00 to 20:00 h) and "night" (22:00 to 06:00 h) periods. Additional illumination level measures were collected hourly (06:00 to 22:00 h) in 20 locations in 2 facilities using hand-held photometers. Measurements were collected in pt rooms, common areas, and outdoor locations accessible to pts. Nighttime noise was further described using structured observations in 25 pt rooms. Documentation included noise loudness, source, location, and description.

Results: Sleep disturbance was common and circadian rhythms were of low amplitude and poor quality. Average daytime illumination in pt rooms was 105 Lx, and was >1000 Lx only 5 min/day. Wrist actigraphy showed pts were exposed to light >1000 Lx an average of 12 min/day. Light measurements showed that light levels were higher in common and outdoor areas than in pt rooms, but pts were seldom found in these locations. At night, there were an average of 17.3 min of noise >60 dB (a level sufficiently loud to disturb sleep)/h, and loud noises were often associated with awakenings from sleep. Based on observations, 53% of nighttime noises were caused by facility staff, 29% by pts, and 18% by other factors. 19 observed noises/h were sufficiently loud to potentially disturb sleep.

Conclusions: Results confirm that both long-stay and short-stay nursing home pts are exposed to very little bright light. Findings add to existing research by demonstrating that light levels in pt rooms are not sufficiently bright to adequately entrain circadian rhythms, and although outdoor spaces with higher illumination levels were available to pts, they were seldom used. During the night, noise levels were exceptionally high, and were associated with awakenings from sleep. Observational data suggested noises were often caused by staff, and that closing room doors and working with facility staff to reduce sleep-disruptive noise may lead to improved pt sleep. Overall, findings suggest facility-level interventions to increase daytime illumination levels and reduce sleep-disruptive nighttime noise should be further explored.

Keywords Nursing home, Light intensity, Noise, Sleep disturbance

Research supported by VA HSR&D IIR 01-053-1 and AIA 03-047, GLAVAHS GRECC, UCLA Claude Pepper OAIC AG-10415 (CDA), and NIA R29 AG13885

EFFECTS OF STRUCTURED SOCIAL AND PHYSICAL ACTIVITY ON SLEEP AND PERFORMANCE IN OLDER ADULTS

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Background: Decreased levels of physical and social activity may contribute to age-related sleep disturbances and cognitive decline. We conducted 2 studies to determine whether exposure to a program of combined structured social and physical activity can have beneficial effects on sleep, circadian rhythms, mood, and neuropsychological performance in elderly residents of retirement communities.

Method: 34 older adults (65 to 92 yrs of age) without unstable medical conditions and either no to mild dementia (MMSE > 24) participated in 2 separate studies. All subjects had 7 days of baseline recordings, 14 days of exposure to an identical program of social and physical activity, and a 2 day post-treatment evaluation. The activity program was performed at the subject's home and consisted of 15 min of warm up and cooling down activities, 45 min of light to moderate physical activity, and 30 min of seated social activity. Rest/activity (actigraphy) and sleep diaries were recorded continuously throughout the study. Polysomnography, core body temperature, assessment of neuropsychological performance, and mood were performed at baseline and again during the 2 days after the intervention. In study 1, 22 subjects were exposed to either the 1.5 h activity program 2 times per day (09:00 to 10:30 and 19:00 to 20:30 h) or to a control condition in which the subjects did not alter their daily activities for 14 days. In study 2, we wanted to determine whether a single morning or evening activity session would be sufficient to improve sleep and performance. Using a cross-over design, 12 subjects completed this study. Subjects were exposed to either the morning (09:00 to 10:30 h) or evening (19:00 to 20:30 h) activity program.

Results: In study 1, exposure to 14 days of both morning and evening activity increased the amount of slow wave sleep and improved memory-oriented tasks. However, no significant changes were seen in the amplitude or phase of the circadian rhythm of core temperature or subjective measures of vigor and mood. In study 2, exposure to either morning or evening activity significantly improved neuropsychological performance. Morning activity sessions increased throughput on 4 of 8 tasks, while evening sessions improved throughput on 7 of 8 tasks. Subjective sleep quality ratings using the Pittsburgh Sleep Quality Index improved following both morning or evening sessions. However, in this second study, objective measures of sleep did not show significant improvement.

Conclusions: The results of these studies demonstrate that short-term exposure to structured social and physical activity programs can improve sleep quality and neuropsychological performance in older adults. While evening activity sessions may have more consistent effects, increasing activity at other times of the day is also beneficial.

Keywords Elderly, Sleep, Bright light, Melatonin, Structured social and physical activity, Morning, Evening

Supported by P01-AG-11412, 5K07 HL03891 and Brookdale National Foundation

POSTER PRESENTATION SESSION K

REACTIVITY OF BLOOD PRESSURE (BP) TO ACTIVITY AT DIFFERENT TIMES OF DAY

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Objectives: BP exhibits a circadian pattern, but it remains unclear to what extent this is mediated by internal factors or changes in activity. Leary et al. (*J. Hypertens*. 2002; 20:865) found the magnitude of increase in activity during the first 4 h after waking was associated with a greater morning surge in BP. Kario et al. (*Hypertension* 1999; 34:658) calculated a BP "reactivity index" to describe the change in BP per unit change in activity, but only general periods of sleep and wakefulness were examined. Our aim was to investigate whether such reactivity of BP to activity changes over a normal sleep-wake cycle.

Methods: Hypertensive pts ($n = 440$) underwent simultaneous 24 h ambulatory BP, heart rate (HR), and activity monitoring. BP and HR were measured every 20 min. Actigraphy scores were averaged over the 15 min preceding a BP measurement. Reactivity indices were calculated for twelve 2 h time periods and then analyzed for time-of-day differences using a repeated-measures general linear model.

Results: Generally, systolic BP and HR were more reactive than diastolic BP. The highest reactivity index for systolic and diastolic BP was

observed between 08:00 and 10:00 h ($p < 0.02$). Between 10:00 and 12:00 h, BP reactivity then decreased ($p < 0.05$). Gender, age, and medication did not affect these circadian changes in BP reactivity.

Conclusions: These results suggest that the reactivity of BP and HR to a given change in activity is highest in the morning when subjects live normally. Our experimental work will now focus on separating the effects of posture and exercise on BP reactivity.

Keywords Systolic blood pressure, Diastolic blood pressure, Heart rate, Activity, Circadian rhythm

AMPA RECEPTOR BLOCKADE AFFECTS CIRCADIAN RHYTHM OF BLOOD PRESSURE (BP) AND HEART RATE (HR) IN RATS

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Objectives: Glutamatergic AMPA receptors are present in the suprachiasmatic nucleus of the hypothalamus, which is the circadian clock of mammals, and also in other brain areas involved in the regulation of cardiovascular functions. Therefore, our goal was to study if AMPA receptors have a role in the regulation of BP and HR and their circadian rhythm.

Methods: GYKI53655 and GYKI53405, 2 non-competitive AMPA antagonists, were applied during the light or dark periods of the day to freely moving Wistar rats ($n = 6$/group) at the intraperitoneal dose of 20 mg/kg. BP, HR, and body temperature (BT) were continuously recorded using a telemetry system (Data Sciences). Artificial illumination started at 06:00 h for 12 h and alternated with 12 h darkness. Statistical analysis was done using a two-way repeated measure ANOVA using the Newman Keuls *post hoc* test.

Results: Both GYKI53405 and GYKI53655 increased BP and HR by 10% to 20% for 3 to 4 h when given in the morning (10:00 h). However, GYKI53405 and GYKI53655 induced either a brief and small hypertension and tachycardia (3% to 8%) or no change in BP and HR if injected in the afternoon (16:00 h, near the beginning of the active period of rats when BP and HR generally start to rise). Moreover, following treatment with GYKI53405 during the subjective night of rats (1 h after lights off), HR and BP remained unaffected. In striking contrast, both compounds caused a similar decrease in BT independently from the time of treatment.

Conclusions: Systemic inhibition of AMPA receptors increased BP and HR to a greater extent during the light than the dark period of the day,

suggesting that AMPA receptors might be involved in the regulation of the circadian rhythm of BP and HR in rats.

Keywords Circadian rhythm, Blood pressure mechanisms, Heart rate, Chronopharmacology, Glutamatergic AMPA receptor blockade

AGE-RELATED CIRCADIAN VARIATIONS OF CAROTIDCARDIAC BAROREFLEX IN SLEEP-DEPRIVED SUBJECTS

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Objectives: The aim of the present study was conducted to determine whether the circadian variations of baroreflex sensitivity as a function of human aging are attributable to changes in the cardiovascular control in the elderly and whether sleep deprivation reduces carotid cardiac baroreflex in humans?

Methods: 12 healthy male subjects 20 to 38 yrs old and 12 healthy male subjects 50 to 60 yrs old were studied. The neck suction method was used to measure carotid baroreceptor activation. The circadian variability of cardiac responses to carotid baroreceptor activation was analyzed in both groups of subjects after normal sleep and after 24 h without sleep. The cardiac response was calculated as the ratio of reflex RR interval prolongation during 5 s of suction to the neck suction stimulus.

Results: In all older subjects a reduction of cardiac response to carotid baroreceptor activation was observed in comparison with the younger volunteers. The circadian course of the cardiac reflex response to baroreceptor activation was shifted downward in this group of subjects. The cardiac response to carotid body activation reached a minimum between 03:00 and 07:00 h in all subjects. Both groups of subjects showed a reduction of baroreflex cardiac control after sleep deprivation, but the cardiac response to baroreceptor activation was especially diminished in the older group.

Conclusions: Thus, an impaired vagal control of cardiac activity might be related to the risk time for the occurrence of cardiac disorders, especially for cardiac arrhythmias. Additionally, sleep deprivation decreases cardiac baroreflex control. The reduction of reflex control of cardiac function after sleep deprivation is more dangerous in older subjects.

Keywords Carotid cardiac baroreflex, Aging, Circadian rhythms, Sleep deprivation

COGNITIVE PERFORMANCE IN CHILDREN AND ADOLESCENTS WITH DELAYED SLEEP PHASE SYNDROME (DSPS)

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Objectives: Children and adolescents with DSPS might experience difficulties in daytime school activities, which sometimes are as severe as in individuals with Attention Deficit/Hyperactivity Disorder (ADHD). The present study examined the pattern of associations between sleep/sleep-wake parameters and cognitive performance in a group of patients (pts) with DSPS and ADHD *vs.* DSPS otherwise healthy.

Methods: 26 pts with DSPS (6 females), mean age of 13.6 ± 3.6 yrs, participated. Half were previously diagnosed with ADHD and were treated with stimulant medications during the study period. The diagnosis of DSPS was established based on clinical interview and actigraphic monitoring of rest-activity patterns. Cognitive performance was examined by administration of Test of Variables of Attention (TOVA).

Results: 10 pts showed a pathologic ADHD score, 4 of which had no previous diagnosis of ADHD. Different patterns of associations between actigraphic and TOVA variables were revealed for pts with and without previously diagnosed ADHD. In the ADHD group, decreased level of performance on several variables of attention and impulsivity was associated with later acrophase of rest-activity cycle ($r = -0.673$ to $r = -0.827$; $p < 0.05$). In pts without ADHD, more restlessness during sleep was associated with more attention decrements ($r = -0.785$ to $r = -0.883$; $p < 0.05$).

Conclusions: The results of this study demonstrate that some young pts with DSPS can manifest ADHD-like impairment in performance, thus being likely to receive a misdiagnosis. Further, cognitive decrements were associated with different characteristics of DSPS in pts with and without ADHD. Impaired performance was related to a more delayed circadian rhythm of the sleep-wake cycle in pts with ADHD; whereas, performance in pts without ADHD was associated with sleep quality.

Keywords Delayed sleep phase syndrome, Attention/hyperactivity disorder, Cognitive performance, Test of variables of attention

BRIGHT LIGHT THERAPY (BLT) IN ELDERLY DEPRESSIVE PATIENTS WITH INSOMNIA: TWO CASE REPORTS

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Objectives: The previous studies support BLT effectiveness on insomnia and depression symptoms in the elderly. In this report we discuss 2 elderly depressive patients (pts) with heavy insomnia symptoms who responded to BRL when added to antidepressant therapy.

Methods: 68 and 73 yr old male pts with insomnia and depressive symptoms were given BLT as an add-on to SSRI antidepressant therapy. Hamilton Rating Scale for Depression (HRSD), Hamilton Anxiety Scale (HAS), sleep diary, and Insomnia Severity Index (ISI) were used to determine the efficiency of the therapy. Light was applied as 10,000 Lx (Philips HF 3305 Bright Light Energy) for 1 h every morning, 5 day/week, for 2 wks.

Results: Case 1: Initial scores (1st day of treatment) were HRSD: 10, HAS: 10, ISI: 24 and final scores (last day of the treatment) HRSD were: 6, HAS: 7, ISI: 10. Case 2: Initial scores (1st day of treatment) were HRSD: 28, HAS: 23, ISI: 28 and final scores (last day of the treatment) HRSD were: 8, HAS: 7, ISI: 14.

Discussion: At the end of the 1st week of treatment there was a clear reduction in depression and anxiety severity and a clear improvement in sleep quality. After discharge, the clinical course of each pt was evaluated for 2 months and documented continuation of the symptom remission.

Conclusion: BLT could be an alternative choice in elderly depressive patients with insomnia symptoms. BLT is safer than classical drug therapies, especially in pts with liver or kidney problems. BLT was found to be an effective add-on antidepressant treatment in our two case studies.

Keywords Bright light therapy, Insomnia, Depression, Elderly, Phototherapy, Insomnia

EVALUATION OF THE TIME-DEPENDENT PROTECTIVE EFFECT OF MELATONIN (M) ON RADIATION-INDUCED DAMAGE OF LUNG TISSUE

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Objectives: The aim was to investigate the effect of M against total body ionizing radiation, given to the rats either in the morning (1 HALO) or evening (13 HALO).

Methods: A total 41 adult male rats synchronized with light-dark cycle (lights on 08:00 h) were randomized to 6 groups (n = 6-7/group). The treatment design is shown in the table:

Group	Time of sacrifice	Irradiation	Ethanol (20%)	Melatonin
1A (Control)	1 HALO[a]	−	−	−
1B (Control)	13 HALO	−	−	−
2A	1 HALO	+	+	−
2B	13 HALO	+	+	−
3A	1 HALO	+	−	+
3B	13 HALO	+	−	+

[a]HALO = Hours after light onset.

M or vehicle (ethanol) were administered immediately before, after, and 24 h preceding irradiation to the rats (M dose: 10 mg/kg, 20 mg/kg, and 100 mg/kg, *i.p.*, respectively). 48 h after irradiation, all animals were sacrificed using ketamine. Changes in malondialdehide (MDA) levels, index of lipid peroxidation, and myeloperoxidase (MPO) activity, index of neutrophil infiltration, were determined in lung tissue. Differences between groups were statistically analyzed by 2-way ANOVA.

Results: In control groups (1A-1B) the MDA levels were found higher at 13 HALO, but MPO activity did not exhibit any significant changes. MDA levels and MPO activity did not change after irradiation at both time points (2A-2B), but M treatment caused a significant decrease in both parameters (3A-3B). After radiotherapy MPO activity was significantly higher at 1 HALO. MDA levels were significantly lower at 1 HALO after M treatment compared to 13 HALO. A significant interaction between time and treatment was determined by 2-way ANOVA.

Conclusions: MPO activity was higher following morning than evening radiotherapy, while MDA levels were not. These results implicate that daytime irradiation causes more neutrophil infiltration. M treatment reduced oxidative damage more prominently in rats receiving morning radiotherapy as determined by MDA levels. The effect of M can be attributed to its well-known antioxidant and free radical scavenger properties.

Keywords Melatonin treatment, Radiation protection, Circadian rhythm, Lung injury, Rats

EFFECT OF HORMON REPLACEMENT THERAPY (HRT) ON POSTMENOPAUSAL BLOOD PRESSURE (BP) PROFILE: A PRELIMINARY STUDY

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Objectives: It is believed that menopause in women increases and postmenopausal HRT decreases the risk of cardiovascular mortality. The aim of this study is to determine the effect of HRT on BP characteristics in normotensive postmenopausal women prospectively.

Methods: 11 normotensive postmenopausal women (mean age 48.5 yrs, median duration of amenorrhea 11 months (mo), plasma level of follicle stimulating hormone 69 $\pm$ 5.3 IU/mL, not receiving HRT the last 6 mo) were studied by 24 h ABPM (Spacelabs 90207) at baseline and after receiving estrogen plus progestagen 12 mo of follow-up. Data were analyzed by ABPM-FIT and CV-SORT and comparisons were made by two-tailed *t*-test.

Results: Although there was a trend of increase in all variables, no significant changes were observed in women taking HRT. Only the rate-pressure product (RPP: systolic BP $\times$ HR) displayed a statistically significant increase ($p = 0.02$) after 12 mo follow-up.

Variable	Before HRT	After HRT
Systolic BP		
24 h mean	113.3 $\pm$ 0.40	115.5 $\pm$ 0.41
Day mean	117.5 $\pm$ 0.41	118.3 $\pm$ 0.42
Night mean	104.1 $\pm$ 0.45	106.4 $\pm$ 0.45
Diastolic BP		
24 h mean	70.7 $\pm$ 0.32	72.3 $\pm$ 0.33
Day mean	74.9 $\pm$ 0.31	75.4 $\pm$ 0.32
Night mean	62.0 $\pm$ 0.36	61.9 $\pm$ 0.37
Heart Rate		
24 h mean	70.3 $\pm$ 0.41	73.4 $\pm$ 0.47
Rate-pressure product		
24 h mean	7813 $\pm$ 68	8401 $\pm$ 76[a]

Conclusion: Despite previous reports of beneficial effects of HRT on ambulatory BP and HR we did not find any change other than an increase in RPP in this prospective study. Therefore, long-term monitoring of BP and HR may be required.

Keywords Hormone replacement therapy, Rate-pressure product, Systolic blood pressure, Diastolic blood pressure

MELATONIN (M) IN COMPLEX THERAPY OF OLD PATIENTS (PTS) WITH POSTINFARCTIONAL CARDIOSCLEROSIS

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Objectives: The aim of the present study is to investigate influence of M on clinical symptoms, morphofunctional disturbances in myocardium, peroxidal oxidation of lipides (POL), and antioxidant defense (AOD) on the background of traditional therapy (TT) of old patients with postinfarctional cardiosclerosis (PIC) and heart failure (HF).

Material and Methods: 40 pts with IHD, PIC, HF, functional class (f.c.) II-IV (by NYHA), stenocardia f.c. II-III were investigated. Among them were 13 men and 27 women. Mean age was 69.9 ± 1.8 yrs. Pts were divided into 2 randomized groups depending on M doses. Pts received TT with nitrates, β-blockers, ACE inhibitors, aspirin, or diuretics. On the background of TT, 20 pts of the 1st group obtained M in a dose of 6 mg at 22:00 h. 20 pts of the second group received M in a dose of 3 mg at 22:00 h with TT. Before and 20 days after therapy ECG-monitoring (Astrocard, Russia), dopler-EchoCG (ESAOTE, Italy), level of malone dialdehide (MDA), and antioxidant enzymes in red cells were examined.

Results: Data obtained testify to expressive antianginal and antiischemic effects of M in different doses. These effects were revealed on 5 ± 0.4 days in the first and on the 4.5 ± 0.3 days in the second groups. In the first and second groups, there were decreased f.c. of HF, number and duration of angina attacks, and increased tolerance of physical load by 55 and 77 meters, respectively. The number of required nitroglycerin tablets was reduced in pts of the 1st group and had tendency to reduction in pts of the second group. In these 2 groups, there were decreased episodes of ST-elevation. The number of episodes of ST-depression was reduced in the 1st group and had tendency to reduction in the second group. End-diastolic and end-systolic sizes and

volumes were also reduced. Ejection fraction (EF) increased in the 1st group (from 52.9 to 55.4%). MDA level declined in both groups. An increase in activity of Cu, Zn-SOD was significant, especially in the first group.

Conclusion: M on the background of TT produced antianginal, antiischemic effects in IHD, PIC, and HF pts, improved clinical symptoms, and reduced or eliminated episodes of ST-depression and ST-elevation.

Keywords Melatonin, Postinfarctional cardiosclerosis, Myocardium, ST-segment depression, ST-segment elevation, Physical exertion

PREDICTORS OF MENSTRUAL FLOW DURATION (MFD) IN TURKISH WOMEN

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Objectives: Few studies investigate correlates of MFD. Longer duration of menstrual flow appears to be associated with dysmenorrhea. One study found longer bleeding to be associated with occurrence type 2 diabetes (DM). We investigated predictors of the recalled duration of menstrual bleeding in a group of Turkish women aged 20 to 30 yrs from Isparta who were interviewed at ages 45 to 60 yrs for a study on menopause.

Methods: Demographic, anthropometric, and reproductive characteristics were assessed by questionnaire. Multiple regression analysis was performed to determine predictors of MFD. A stepwise model selection algorithm was applied with a significance level of 0.15 for inclusion of variables into the model.

Results: In univariate analysis, presence of dysmenorrhea ($p = 0.0212$), shorter cycle length ($p = 0.0098$), and lower body weight at 25 yrs of age ($p = 0.0022$) was associated with longer MFD. In multivariate analysis the above factors, except for cycle length ($p = 0.0523$), remained significant and yr of birth also emerged as a significant predictor ($p = 0.0395$). Later yr of birth was associated with shorter MFD. Limitations of this study are menstrual characteristics were self-reported and retrospectively assessed. Nevertheless inaccuracies in recall are expected to diminish rather than cause the observed associations.

Conclusions: Most of current findings are consistent with previous research. A possible association between yr of birth and MFD has not

been before reported and suggests secular influences on reproductive characteristics. Prospective studies on this issue are warranted.

BODY TEMPERATURE RHYTHM IN THE ELDERLY: EFFECT OF SINGLE DAILY MELATONIN (M) DOSES

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Objectives: The present study is part of a more extensive investigation dedicated to age-dependent changes/disturbances of the circadian system and their treatment.

Methods: 97 subjects of both genders between 65 to 91 yrs of age participated; 63 were assigned to M treatment and 34 to P (placebo). Study was performed in the Tyumen Elderly Veteran House in February to April, 2003, and February to March, 2004. Every participant lived at sleep-wake regimen of personnel convenience without any researcher-applied restrictions to preserve age-specific lifestyle. The experiment lasted for 3 weeks (1 wk control and 2 wks of treatment). Body temperature (BT) was measured using calibrated mercury thermometers at 03:00, 08:00, 11:00, 14:00, 17:00, and 23:00 h each day of the 1st and the 3rd wk. Specially trained personnel took the measurements avoiding disturbing subject's sleep. The M-treated subjects received a melatonin dose of 1.5 mg (Melaxen^{TM}) and the P-treated subjects received placebo each day of the 2nd and the 3rd wks at 22:30 h. To evaluate age-dependent changes the data of an earlier experiment performed on 120 young adults (17 to 39 yrs of age) under similar conditions were used. Rhythm characteristics were estimated by means of Cosinor analyses and intra- and inter-individual variability in mean values by ANOVA.

Results: The circadian amplitude of BT was slightly decreased in elderly subjects prior to M treatment (0.26 ± 0.01°C) as compared to young adults (0.33 ± 0.01°C). The mean circadian acrophase was similar in both age groups (16.93 and 17.07 h, respectively). However, the inter-individual differences were higher in the older group. The single values varied between 10:00 and 23:00 h. Under M treatment, the mesor was lower by 0.1°C, and the amplitude increased to 0.34 ± 0.01°C, a similar value as in young adults. This was probably due to the increase of the inter-daily rhythm stability. The mean acrophase did not change under M treatment, and the inter-individual variability decreased considerably (individual values between 13:00 and 20:00 h). A highly significant

correlation between the acrophase before treatment and the phase change under treatment indicates a synchronizing effect of M.

Conclusions: The data clearly show that age-dependent changes mainly concern rhythm stability and the synchronization with the 24 h day. M exerts direct (hypothermic) and chronobiotic (synchronizing) effects.

Keywords Body temperature, Circadian rhythm, Age-dependent changes, Melatonin, Rhythm stability, Synchronization

TEMPORAL VARIATION IN THE INTERACTION BETWEEN CAFFEINE (C) AND MORPHINE (M)-INDUCED ANALGESIA

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Objective: Caffeine, an adenosine A_1, A_2, and A_{2B} receptor antagonist, is used as an adjuvant drug in combination with opioids or nonsteroid anti-inflammatory drugs. The aim of this study was to determine any circadian variation in the interaction between C and M-induced analgesia in the hot-plate test.

Methods: Local bred albino male mice (35 to 45 g) synchronized with a standard light-dark regimen (12 h:12 h L:D, lights on 08:00 h) were used. Basal pain sensitivity (saline; *i.p.* 10 ml/kg) and M-induced analgesia (10 mg/kg *i.p.* 30 min before hot-plate test; 55 $\pm$ 0.5°C) were determined in groups of 10 to 12 mice at 1, 5, 9, 13, 17, and 21 HALO (Hours After Light Onset). To test the administration time-dependent interaction, a 3rd group of animals were injected with a predetermined dose of C (20 mg/kg) 30 min before M administration, and response latencies were tested at the same time points. In all tests, response latencies shorter than 3 sec were regarded as to being spontaneous and excluded. To avoid heat injury, animals not responding within 45 sec were removed from the hot-plate (cut-off time). All the experiments were performed between January and April to avoid seasonal variations. Data were expressed as% absolute change (%AC) with regard to the time-dependent efficacy of M and analyzed by ANOVA or Students' t-test where necessary.

Results: Saline-injected control groups displayed a circadian rhythmicity in hot-plate response latency with higher values during the activity period. A similar pattern was observed for the analgesic activity of M. C alone (10 mg/kg) did not cause any significant change in hot-plate response latencies when applied 60 min before the test compared to

saline-injected animals. Pretreatment C potentiated M-induced antinociceptive activity significantly at HALO 1 and HALO 9 in terms of %AC, but not at other time points.

Conclusion: The results of this study indicate that there is administration time-dependent interaction between C and M-induced analgesia in mouse hot-plate test. The findings of this study should be considered for the adjuvant use of C with analgesics.

Keywords Caffeine, Morphine-induced analgesia, Time-dependent variations, hot-plate test, Pain, Mice, Circadian rhythm

TEMPORAL VARIATION IN THE INTERACTION BETWEEN LITHIUM CHLORIDE (LC) AND MORPHINE (M)-INDUCED ANALGESIA

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Objective: Phosphoinositide pathways are important determinants of opioid-induced antinociception. It has been reported that LC pretreatment 18 h before the testing reduces the analgesic effect of M, by interfering with phosphotidylinositol turnover. The aim of the present study was to determine if there is a circadian variation in the interaction between LC and M-induced analgesia in mouse hot-plate test.

Methods: Local bred albino male mice (35 to 45 g) synchronized with a standard light-dark regimen (12 h:12 h L:D, lights on 08:00 h) were used. Basal pain sensitivity (saline; *i.p.* 10 mL/kg) and M-induced analgesia (10 mg/kg *i.p.* 30 min before hot-plate test, 55 $\pm$ 0.5°C) were determined in groups of 10 to 12 mice at 1, 5, 9, 13, 17, and 21 HALO (Hours After Light Onset). To test the administration time-dependent interaction, a 3rd group of animals was injected with a predetermined dose of LC (0.3 mmol/kg) 30 min before M treatment, and response latencies were tested at the same time points. Response latency shorter than 3 sec was regarded as to being spontaneous and excluded. To avoid heat injury, animals not responding within 45 sec were removed from the hot-plate (cut-off time). All the experiments were performed between January and April to avoid seasonal variations. Data were expressed as % absolute change (%AC) with regard to the time-dependent efficacy of M and analyzed by ANOVA or Students' *t*-test where necessary.

Results: Saline-injected control groups displayed a circadian rhythmicity in hot-plate response latency with higher values during the activity period. A similar pattern was observed for the analgesic activity of M. LC

alone (1 to 10 mmol/kg) did not cause any significant change in hot-plate response latencies when applied 60 min before the test compared to saline-injected animals. Pretreatment with LC did not affect M-induced antinociceptive activity at most time points, except HALO 9 when there was a prominent potentiation in M analgesia in terms of %AC.

Conclusions: The results of this study indicate that short-term (30 min before hot-plate test) and long-term (18 h before the test) effects of LC on M-induced antinociceptive activity are quite different. This administration-time-dependent interaction between LC and M-induced analgesia should be considered in studies aimed to analyze the involvement of LC on nociceptive processes.

Keywords Lithium, Morphine-induced analgesia, Time-dependent variations, Hot-plate, Mice, Pain, Circadian rhythm

ADMINISTRATION TIME-DEPENDENT MODULATORY EFFECT OF SODIUM VALPROATE (SV) ON MORPHINE (M)-INDUCED ANALGESIA IN MICE

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Objective: Substances that modulate GABAergic activity can affect opiate-induced analgesia. SV, an anticonvulsant and mood stabilizer, increases extracellular GABA levels in the CNS by activation of glutamic acid decarboxylase and inhibition of GABA transaminase. The purpose of present study is to evaluate administration-time-dependent interaction between SV and M-induced analgesia.

Methods: Local bred albino male mice (35 to 45 g) synchronized with a standard light-dark regimen (12 h:12 h L:D, lights on 08:00 h) were used. Basal pain sensitivity (saline; *i.p.* 10 ml/kg) and M-induced analgesia (10 mg/kg i.p. 30 min before hot-plate test, 55 $\pm$ 0.5°C) were determined in groups of 10 to 12 mice at 1, 5, 9, 13, 17, and 21 HALO (Hours After Lights On). To test the administration time-dependent interaction, a 3rd group of animals was injected with a predetermined dose of SV (10 mg/kg) 30 min before M, and hot-plate response latencies were tested at the same time points. A response latency shorter than 3 sec was regarded as spontaneous and excluded. To avoid heat injury, animals not responding within 45 sec were removed from the hot-plate (cut-off time). All the experiments were performed between January and April to avoid seasonal variations. Data were expressed as% absolute change (%AC) with regard to the

time-dependent efficacy of M and analyzed by ANOVA or Student's *t*-test as necessary.

Results: Saline-injected control groups displayed circadian rhythmicity in hot-plate response latency with higher values during the activity period. A similar pattern was observed for M analgesic activity. SV alone (10 to 40 mg/kg) did not cause any significant change, compared to saline-injected animals, in hot-plate response latencies when applied 60 min before test. Pretreatment with SV caused significant potentiation of the M analgesic activity at HALO 1, 9, and 13 in terms of the %AC.

Conclusions: Our study reveals administration-time-dependent interaction between SV and M-induced analgesia in the mouse hot-plate test. The well-known circadian variation in activity of the enzymes involving GABA turnover may be responsible for this temporal interaction pattern.

Keywords Sodium valproate, Morphine-induced analgesia, Time-dependent variations, Circadian rhythm, Hot-plate, Mice

TEMPORAL VARIATION IN THE INTERACTION BETWEEN AGMATINE (A) AND MORPHINE (M)-INDUCED ANALGESIA

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Objective: A is an endogenous cationic amine reported to enhance M-induced analgesia among its diverse biological effects. The aim of this study was to determine if there is circadian variation in the interaction between A and M-induced analgesia in the hot-plate test.

Methods: Local bred albino male mice (38 to 45 g) synchronized with a standard light-dark regimen (12 h:12 h L:D, lights on 08:00 h) were used. Basal pain sensitivity (saline; *i.p.* 10 ml/kg) and morphine-induced analgesia (10 mg/kg i.p. 30 min before hot-plate test; $55 \pm 0.5^{\circ}$C) were determined in groups of 10 mice at 1, 5, 9, 13, 17, and 21 HALO (Hours After Light Onset). To test the administration-time-dependent interaction, a 3rd group of animals was injected with a predetermined dose of A (40 mg/kg) 30 min before M, and response latencies were tested at the same time points. Response latency shorter than 3 sec was regarded as to being spontaneous and excluded. To avoid heat injury, animals not responding within 45 sec were removed from the hot-plate (cut-off time). All the experiments were performed during the winter in January and February to avoid seasonal variations. Data were expressed as %

absolute change (%AC) with regard to the time-dependent efficacy of M and analyzed by ANOVA or Student's *t*-test as necessary.

Results: Saline-injected control groups displayed a circadian rhythm in hot-plate response latency with higher values during the activity period. A similar pattern was observed in M-induced analgesic activity. A (40 mg/kg) when applied alone 60 min before the test did not cause significant change in hot-plate response latencies compared to saline-injected animals. Pretreatment with A did not affect M-induced antinociceptive activity at most time points but reduced M-induced antinociceptive activity significantly at HALO 5 in terms of %AC.

Conclusion: This administration-time–dependent interaction between A and M-induced analgesia should be considered in studies aimed at analyzing the involvement of A in nociceptive processes.

Keywords Agmatine, Morphine-induced analgesia, Time-dependent variations, Hot-plate, Circadian rhythm, Mice

CIRCADIAN VARIATIONS OF MEAN ARTERIAL BLOOD PRESSURE (MAP) IN OBESE SHIFT WORKERS

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Objectives: Several studies indicate the elevated occurrence of cardiovascular diseases in shift workers. Obesity is a risk factor of arterial hypertension. We studied circadian variation of MAP in healthy men with normal body mass and in obese men after night work and if reduction of body mass changes the circadian variation of MAP.

Methods: 20 healthy male shift workers 40 to 50 yrs old (control group) and 12 obese age-matched shift workers were study. MAP was measured and analyzed during 24 h after the day shift and compared with the values of MAP after the night shift. In obese subjects the same investigation was performed after body mass reduction to the normal. 24 h blood pressure monitoring was performed during constant routines in a "chronobiological bunker": subjects were kept in a constant climate on continuous bed rest for 24 h while maintained on a low-protein diet.

Results: An increase in MAP was observed in all subjects during the 24 h following the night in comparison with the day work. This reaction was significantly augmented in obese subjects; all obese subjects and 2

controls showed MAP values above threshold for the diagnosis of hypertension. However, after body mass reduction, the increase in MAP after night work was similar to the increase observed after night work in the control group (normal body mass). A maximal value of MAP after weight reduction was 87% of the maximal MAP value before weight reduction (in the obese group). This difference was significant ($p < 0.01$).

Conclusions: Night work, as a disturbance of the normal sleep-wake rhythm, may produce an increase in MAP. In obese subjects, night work might be a potential risk factor for arterial hypertension. Body mass reduction might prevent development of arterial hypertension in obese night workers.

Keywords Shift work, Arterial hypertension, Obesity, Body mass reduction, Mean arterial blood pressure

DIFFERING EFFECTS OF AWAKENING VERSUS BEDTIME VALSARTAN (V) ADMINISTRATION ON AMBULATORY BLOOD PRESSURE IN ELDERLY HYPERTENSIVE PATIENTS

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Objectives: Previous studies indicate V administration at bedtime, as opposed to upon wakening, improves the day/night ratio of BP towards a more dipper pattern, without loss in 24 h efficacy (*Hypertension* 2003;42:283–290). This ratio is characterized by a progressive decrease with aging. Accordingly, we investigated the administration-time-dependent efficacy of V in elderly hypertensive patients.

Methods: We studied 97 elderly patients with grade 1 to 2 essential hypertension (37 men), 66.1 ± 0.7 yrs of age, randomly assigned to take V (160 mg/day) as a monotherapy either upon awakening or at bedtime. BP was measured by 48 h ambulatory monitoring at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after 3 months (mo) of therapy.

Results: There was a highly significant BP reduction after 3 mo of V ($p < 0.001$), slightly larger after bedtime dosing (13.3 and 7.1 mm Hg reduction in the 24 h mean of systolic and diastolic BP with V taken upon awakening; 15.4 and 9.4 mm Hg when V was taken at bedtime).

The day/night BP ratio was unchanged when V was taken upon awakening (−1.3 and −0.1 for systolic and diastolic BP; $p > 0.171$). This ratio was significantly increased (7.5 and 6.8 for systolic and diastolic BP; $p < 0.001$) when V was taken at bedtime. The reduction of nocturnal mean was doubled when V was taken at bedtime *vs.* morning ($p < 0.001$).

Conclusions: In elderly hypertensive pts, mainly characterized by a diminished nocturnal decline in BP, bedtime dosing of V might be advantageous for improved efficacy during the nocturnal resting hours, and the potential reduction in cardiovascular risk associated with a normalized BP day/night ratio.

Keywords Valsartan, Elderly, Chronotherapy, Day/night blood pressure ratio, Elderly hypertensive patients

GENDER-DIFFERENCES IN THE ADMINISTRATION-TIME-DEPENDENT EFFICACY OF VALSARTAN (V) ON AMBULATORY BLOOD PRESSURE (BP)

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Objectives: Previous investigations have shown gender differences in the pathophysiology of hypertension and treatment responses to medication. However, comparative efficacy studies of angiotensin receptor blockers (ARB) according to gender are uncommon. An administration-time-dependent effect on BP has been documented for valsartan (*Hypertension* 2003;42:283–290). This investigation explores the antihypertensive efficacy of V according to gender and time of treatment.

Methods: We studied 101 men and 178 women with grade 1 to 2 essential hypertension, 50.1 ± 13.3 yrs of age, randomly assigned to take V monotherapy (160 mg/day) either upon awakening or at bedtime. BP was measured for 48 h by ambulatory monitoring at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night, before and after 3 months (mo) of therapy.

Results: There was a highly significant BP reduction after 3 mo of V therapy ($p < 0.001$), similar for men and women at both treatment times (14.5 and 10.4 mm Hg in the 24 h mean of systolic and diastolic BP in men with V ingestion upon awakening, 14.9 and 10.2 mm Hg in women, $p > 0.830$ among genders; 14.2 and 10.7 mm Hg in men with

ingestion upon V at bedtime, 15.8 and 10.8 in women, $p > 0.289$). The day/night BP ratio was unchanged with V upon awakening. This ratio was significantly increased (6.5 and 6.7 for systolic and diastolic BP in men, 4.8 and 4.5 in women; $p > 0.107$ for gender differences) with V administered at bedtime.

Conclusions: Independent of the time of administration, 160 mg/day V efficiently reduces BP for the entire 24 h and to a similar extent in both men and women. The differing administration-time–dependent effects of V on the day/night BP ratio should be taken into account when prescribing this ARB to non-dipper hypertensive patients.

Keywords Valsartan, Gender differences, Chronotherapy, Non-dipper hypertensives, Blood pressure

VALSARTAN (V) MONOTHERAPY EFFICIENTLY REDUCES AMBULATORY PULSE PRESSURE (PP) IN HYPERTENSIVE PATIENTS (PTS)

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Objectives: Increased PP has been identified as an independent marker of cardiovascular risk, mainly for myocardial infarction, congestive heart failure, and cardiovascular death. Previous studies have concluded that angiotensin-II receptor blockers differ in their ability to reduce PP (*J. Hypertens.* 2000;18:1683–1690). Accordingly, we investigated the effects of V on ambulatory PP in pts with essential hypertension.

Methods: We studied 318 pts (pts) with grade 1 to 2 essential hypertension (113 men and 205 women), 50.1 ± 13.5 yrs of age, assigned to take V (160 mg/day) as a monotherapy. Blood pressure (BP) was measured for 48 h by ambulatory monitoring at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after 3 months (mo) of treatment.

Results: V treatment resulted in a highly statistically significant reduction of BP from baseline (15.0 and 10.6 mm Hg reduction in the 24 h mean of systolic and diastolic BP, respectively; $p < 0.001$). There was a highly significant reduction ($p < 0.001$) of 4.4 mm Hg in the 24 h mean of PP. The reduction in PP after treatment was highly significant

(after correcting for multiple testing) for each of the 24 hourly means (always $p < 0.001$). Accordingly, the impact of treatment was similar on the diurnal and the nocturnal means of PP (reduction of 4.4 and 4.3 mm Hg, respectively).

Conclusions: V significantly reduces PP homogenously during the 24 h. Apart from the progressive rise of PP with aging, a significant increase in PP has been documented, among other groups of interest, in hypertensive pts with type 2 diabetes. Thus, V could be chosen for added cardiovascular risk reduction in the treatment of elderly and of hypertensive pts with diabetes, an issue that deserves further prospective investigation.

Keywords Valsartan, Pulse pressure, Circadian rhythm, Chronotherapy, Hypertension

EFFECTS OF NEBIVOLOL (N) ON AMBULATORY BLOOD PRESSURE (BP) IN SMOKER AND NON-SMOKER HYPERTENSIVE PATIENTS (PTS)

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Objectives: Cigarette smoking may attenuate the efficacy of antihypertensive medications, mainly beta-blockers, alpha-blockers, and diuretics. Not all beta-blockers (including propranolol and oxprenolol) are indicated for the treatment of hypertensive smokers. We investigated the antihypertensive efficacy of another beta-blocker, N, in cigarette smokers.

Methods: We studied 94 previously untreated pts with grade 1-2 essential hypertension (48 men), 42.1 ± 11.7 yrs of age. 48 pts were non-smokers, 23 were smokers, and 23 were ex-smokers for at least 1 yr. Pts were assigned to take N monotherapy (5 mg/day) upon awakening. BP was measured by ambulatory monitoring for 48 h at 20 min intervals between 07:00 to 23:00 h and at 30 min intervals over night, before and after 8 wks of therapy.

Results: N monotherapy resulted in significant BP reduction from baseline, similar for all groups of pts (12.5, 13.8, and 11.3 mm Hg reduction in the 24 h mean of systolic BP in non-smokers, ex-smokers, and smokers, respectively; $p = 0.591$ for group comparison; 11.0, 11.7, and 10.1 mm Hg reduction in the 24 h mean of diastolic BP; $p = 0.625$).

In all groups, the effects of N were significantly larger on the diurnal than nocturnal mean of BP ($p < 0.001$), resulting in a significant reduction in day/night BP ratio (average reduction of 3.2 and 2.7 in the systolic and diastolic BP ratio for all pts; $p < 0.001$). There was also a significant reduction of heart rate (9.9 beats/min; $p < 0.001$) and of double product (or pressure-rate product) by 2130 mm Hg × beats/min ($p < 0.001$) after treatment.

Conclusions: N in a single morning dose of 5 mg/day efficiently reduces BP, heart rate, and pressure-rate product (an index of myocardial ischemia) in hypertensive pts. As opposed to non-selective beta-blockers, the efficacy of N does not seem to be attenuated by cigarette smoking.

Keywords Nebivolol, Smoking, Extreme-dipper, Hypertension, Pressure-rate product

ADMINISTRATION-TIME-DEPENDENT EFFECTS OF NEBIVOLOL (N) ON AMBULATORY BLOOD PRESSURE (BP) IN HYPERTENSIVE PATIENTS (PTS)

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Objectives: N is a lipophilic beta-blocker without sympathomimetic activity, but with apparently nitric oxide-mediated vasodilating effects. The potential differing effects of N as a function of dosing time has not been evaluated. Accordingly, we investigated the administration-time–dependent antihypertensive efficacy of N.

Methods: We studied 82 pts with grade 1 to 2 essential hypertension (32 men), 45.1 ± 12.3 yrs of age, randomly assigned to take N monotherapy (5 mg/d) either upon awakening or at bedtime. BP was measured for 48 h at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night, at baseline and after 8 and 16 weeks of treatment.

Results: After the initial 8 weeks of treatment, the efficacy of N was slightly but not significantly larger with morning dosing (13.9 and 12.2 mm Hg reduction in the 24 h mean of systolic and diastolic BP with N upon awakening; 12.2 and 9.7 mm Hg with bedtime N dosing; $p > 0.064$ between groups). At both treatment-times, efficacy was significantly larger on the diurnal than nocturnal BP mean ($p < 0.029$). N was equality effective whether taken upon awakening or at bedtime in

reducing the 24 h mean of heart rate (9.5 beats/min). After the second 8 wks of treatment with the same dose, efficacy remained unaltered: there was no added BP reduction. Morning and evening dosing provided similar 24 h reduction from baseline, and BP reduction was significantly better during the day (diurnal BP mean) than night (nocturnal rest BP mean).

Conclusions: Results indicate that 5 mg/d N efficiently reduce BP and heart rate independent of its administration time. At both dosing times here investigated, N reduced the diurnal BP mean to a significantly larger extent than the nocturnal BP mean.

Keywords Nebivolol, Chronotherapy, Circadian rhythm, Hypertension, Blood pressure, Heart rate

ADMINISTRATION TIME-DEPENDENT EFFECTS OF AMLODIPINE (A) ON AMBULATORY BLOOD PRESSURE (BP) IN HYPERTENSIVE PATIENTS (PTS)

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Objectives: A has been shown to be effective in reducing BP throughout the day and night when given once daily. However, the potential chronopharmacologic differing effects of A on BP have been little studied. We investigated the administration-time–dependent antihypertensive efficacy of A in essential hypertension pts.

Methods: We studied 194 pts with grade 1 to 2 essential hypertension (101 men), 55.3 ± 12.1 yrs of age, randomly assigned to take a single daily dose of A (5 mg/d) either upon awakening or at bedtime. BP was measured for 48 h at 20 min intervals from 07:00 to 23:00 h and at 30 min intervals over night, before and after 3 months (mo) of therapy.

Results: BP reduction with A was slightly, but not significantly, larger with bedtime dosing (10.4 and 7.3 mm Hg reduction in the 24 h mean of systolic and diastolic BP with A upon awakening; 11.5 and 7.0 mm Hg with bedtime dosing; $p > 0.463$ for treatment-time effect). The day/night BP ratio (calculated as the nocturnal decline of BP relative to the diurnal mean) was slightly reduced for diastolic BP with morning treatment (-1.6; $p = 0.037$), but remained unchanged after bedtime dosing of A (increase of 0.2 and 0.7 in the day/night ratio of systolic and diastolic BP; $p > 0.337$).

Conclusions: This trial demonstrated that A efficiently reduces BP for the entire 24 h, independent of its administration time with respect to the rest-activity cycle of each individual pt. The similar BP reduction observed in the diurnal and nocturnal BP means indicates that A does not modify the circadian pattern of BP variability, even if administered at bedtime.

Keywords Amlodipine, Chronotherapy, Circadian rhythm, Hypertension

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INSTRUCTIONS TO CONTRIBUTORS

Aims and Scope

Chronobiology International is the journal of biological and medical rhythm research. It is a transdisciplinary journal focusing on biological rhythm phenomena of all life forms. It is the major forum of communications for and among chronobiologists. The journal publishes groundbreaking articles on genetic and molecular mechanisms of biological timekeeping; melatonin and pineal gland rhythms; the chronobiology and chronotherapy of sleep, cardiovascular, pulmonary, and other medical conditions; applied areas such as shift work; plus authoritative review papers, short communications of work in progress, case studies, and letters to the editor. Articles in the journal pertain to basic and applied chronobiology, and to methods, statistics, and instrumentation for biological rhythm study. *Chronobiology International* is the official journal of the International Society for Chronobiology, the American Association for Medical Chronobiology and Chronotherapeutics, and the Society for Light Treatment and Biological Rhythms. The journal is published bimonthly in English.

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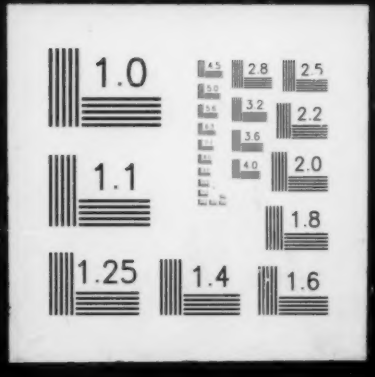
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